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CAUSES OF VARIABILITY IN THE PHARMACOKINETICS OF THROMBOLYTIC DRUGS

J.M.Th. van Griensven

CAUSES OF VARIABILITY IN THE PHARMACOKINETICS OF THROMBOLYTIC DRUGS

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
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GEBOREN TE RIJSWIJK IN 1963

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STELLINGEN

1. Verschillen in leverbloeddoorstroming van patiënten met een acuut myocard infarct bepalen voor een groot deel de variabiliteit in plasma concentraties van zowel alteplase als saruplase.
Dit proefschrift

2. De observaties na veranderingen in leverbloeddoorstroming veroorzaakt door een inspanningsproef of de inname van voedsel suggereren dat onafhankelijke mechanismen de plasma spiegels van t-PA en u-PA reguleren.
Dit proefschrift

3. Het is verbazingwekkend dat van een geneesmiddel als t-PA, dat al jaren op de markt is, de pharmacokinetische eigenschappen nu pas zorgvuldig worden uitgezocht, terwijl deze bij een regulier geneesmiddel al in een veel eerder stadium worden bestudeerd.
Dit proefschrift

4. Complexvorming van t-PA met C1-remmer en α_2-antiplasmine draagt niet bij aan de waargenomen variabiliteit in steady state plasma concentraties van t-PA.
Dit proefschrift

5. Na het infunderen van een lage dosis wordt t-PA in een nog onbekend compartiment gebonden welke mogelijk uit het endotheel kan bestaan.
Dit proefschrift

6. Een homosexueel paar zou bij de procedure voor het adopteren van een kind een gelijke behandeling moeten krijgen als een heterosexueel paar.

7. De maatschappelijke interesse in een onderwerp waarover via een referendum zou worden gestemd, moet dermate breed zijn dat de uitslag pas bij een zeer hoog opkomstpercentage geaccepteerd zou kunnen worden.

8. "The truth is rarely pure and never simple".
Oscar Wilde, The importance of being earnest.

9. Nu er een moskee in Rome verrezen is, zou een kathedraal in Mekka niet misstaan.

10. "Hoe duidelijker je deze wereld ziet, des te sterker ben je genoodzaakt om te doen of zij niet bestaat".
John Irving, Een zoon van het circus.

11. Mensen die leven volgens het devies: "Look like the innocent flower, but be the serpent under it", zullen net als Macbeth hun ambities weldra zien mislukken en een ongelukkig leven leiden.

Stellingen behorende bij het proefschrift: Causes of variability in the pharmacokinetics of thrombolytic drugs.

J.M.Th. van Griensven

Aan mijn ouders en Hanneke
Voor Aarnout

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GENERAL INTRODUCTION

Early use of thrombolytic drugs

Introduction

In 1959 an infusion of highly purified streptokinase was used for the treatment of patients with acute myocardial infarction.[1] The feasibility of using intravenous thrombolytic therapy in these patients was demonstrated for the first time. A year earlier evidence was presented that the primary mechanism of thrombolysis in humans involved the diffusion of plasminogen activator into the thrombus, activation of intrinsic thrombus plasminogen and thrombus dissolution.[2] In the following years the use of fibrinolytic agents in the treatment of acute coronary thrombosis seemed to offer promising results[3], although the importance of coronary thrombosis in the pathogenesis of acute myocardial infarction was still a subject of controversy.[4] Hypotheses regarding alterations of the coronary arteries during early transmural myocardial infarction included ulceration of atherosclerotic plaques with or without subsequent thrombosis, subintimal haemorrhage into a fibrous or softened plaque in the absence of thrombosis, rupture of a softened plaque with progressive occlusion of the coronary lumen, coronary spasm, coronary embolism, and gradual reduction of blood flow to the myocardium secondary to the infarction process itself. The importance of thrombolysis as a treatment of evolving myocardial infarction was recognized only after the demonstration in 1983 by DeWood et al.[5] that the frequency of coronary thrombosis was high during the early hours of transmural myocardial infarction, and that coronary thrombosis was the final common pathway that converted chronic coronary disease to acute myocardial infarction.[6]

The ideal thrombolytic drug

All thrombolytic drugs act on the fibrinolytic system by enhancing the proteolytical degradation of fibrin by the serin protease plasmin (Fig. 1). In addition to this common feature, there are other requirements for the ideal agent to treat patients with acute myocardial infarction. The primary therapeutic aim of thrombolysis is the early reperfusion of a thrombosed coronary artery. Many patients may require surgery or percutaneous transluminal coronary angioplasty (PTCA) to correct residual high grade stenosis soon after administration of the medication and this determines the ideal properties of thrombolytic agents. Systemic administration should be as effective as intracoronary administration because coronary catheterization has its unavoidable morbidity, delay, and high costs. The agent should be nonallergenic, clot-selective, rapidly cleared or inactivated so that haemostasis can be restored promptly when invasive procedures are required and should not produce a generalized breakdown of the haemostatic system, the lytic state, which would increase the chance of major bleeding complications. The search for such an agent was difficult and the ideal

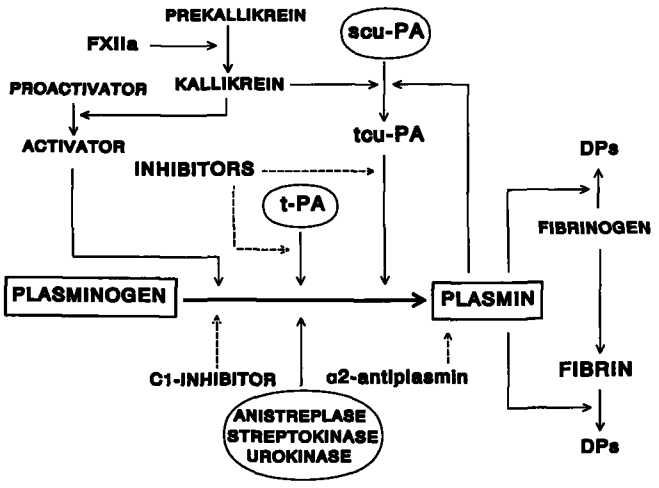

Figure 1. Schematic representation of the fibrinolytic system with the activating pathways of plasminogen to plasmin (——) and site of action of inhibitors (- - -).
FXIIa = factor XIIa, DPs = Degradation products.

thrombolytic drug has not been found. However, streptokinase, anistreplase, urokinase, pro-urokinase and tissue-type plasminogen activator all possess a number of suitable pharmacokinetic and pharmacodynamic properties.

Streptokinase

Streptokinase is a nonenzymatic protein isolated from the broth of Lancefield group C strains of beta-haemolytic streptococci. It is a single polypeptide chain without carbohydrates, with a molecular weight of 47 kilodalton.[7] Streptokinase is an indirect activator of plasminogen. It initially forms a complex with the zymogen plasminogen, which thereby undergoes a transition, allowing exposure of the active site within plasminogen without apparent peptide bound cleavage.[8,9] This modified streptokinase-plasminogen complex behaves as a plasminogen activator.[10] The activator complex can also induce a limited proteolysis in the plasminogen molecule of other streptokinase-plasminogen molecules whereby plasminogen is converted to plasmin. Streptokinase progressively degrades to smaller fragments, resulting in a gradual loss of activator activity.

Streptokinase is cleared from plasma largely by the liver. After injection an immediate rapid clearance phase (half-life of 18 minutes) is followed by a slower disappearance phase (half-life of 83 minutes).[11] The initial rapid phase is probably due to the formation of immune complexes of streptokinase. Human plasma may contain variable levels of antibodies directed against streptokinase,

probably as a result of previous infections with beta-haemolytic streptococci. Sufficient amounts of streptokinase must be infused to neutralize the antibodies before fibrinolytic activation is obtained.[12]

A high initial dose of streptokinase will be associated with a rapid reduction in the level of circulating plasminogen, α_2-antiplasmin and fibrinogen. Excessive lowering of the concentrations of these blood coagulation components in plasma, combined with the inhibitory effect of some fibrinogen degradation products on platelet aggregation and fibrinogen polymerization, are considered responsible for the potentially dangerous bleeding tendency.[13] Rapid infusion of streptokinase can lead to a substantial reduction in blood pressure, probably by excessive activation of the kallikrein-kinin system.[14] After administration of streptokinase antibodies to the thrombolytic agent are formed and this probably precludes repeated administration.[15]

Anistreplase

Anisoylated plasminogen streptokinase activator complex (APSAC) or anistreplase is a streptokinase-plasminogen complex with an anisoyl group reversibly placed within the catalytic centre of the plasmin moiety.[16] Because the catalytic centre is functionally separate from its fibrin-binding site located in the heavy or alpha-chain of plasmin, acylated streptokinase-human plasminogen is catalytically inert so that it can circulate in the vascular system without reaction with either plasma inhibitors or plasminogen, but still binds to fibrin through the unmodified kringle domains of the plasminogen moiety. After activation anistreplase largely behaves like streptokinase, although anistreplase does not cause unacceptable hypotension after an intravenous bolus injection. Compared to streptokinase it has an improved lytic potency and semi-selectivity for clots, causing less fibrinogenolysis. It is metabolised by the liver and has a half-life of 70 ± 23 minutes.

Urokinase

The presence of fibrinolytic activity in urine was discovered in 1913.[17] Much later it was found that a factor in the urine activates plasminogen. Urokinase has been isolated and purified from urine and later from human embryonic kidney cells[18] and certain tumor cells.[19] The gene coding for urokinase has been cloned and expressed in Escherichia coli.[20] Urokinase is a trypsin-like protease composed of two polypeptide chains (20 and 30 kilodaltons) connected by a single disulphide bridge. Urokinase may occur in a high molecular weight form and its proteolytic product or a low molecular weight form that contains mainly the heavy chain. In contrast to streptokinase, urokinase is a direct activator of plasminogen and cleaves a single Arg 560-Val 561 bound in the plasminogen

molecule.[10] The advantages of urokinase isolated from human urine are the absence of immunologic and allergic reactions upon infusion. The mean half-life of urokinase in humans is 14 ± 6 minutes.[21] It is rapidly removed from the blood by clearance and degradation in the liver. Like streptokinase it lacks fibrin specificity. Current interest in urokinase is limited since it rapidly induces a systemic lytic state and is relatively expensive.[22]

Tissue-type plasminogen activator

Tissue-type plasminogen activator (t-PA) has been isolated and purified for the first time from human uterine tissue[23] and a human melanoma cell line.[24] In 1983 bacterial clones containing human t-PA cDNA sequences were identified. A plasmid was constructed for expression in E. coli and a polypeptide was produced with similar fibrinolytic properties as authentic human t-PA.[25] Tissue-type plasminogen activator is a single-chain serine protease with a molecular weight of approximately 70 kilodaltons. The concentration of t-PA in human plasma is approximately 5 ng/ml. Purified t-PA binds specifically to fibrin.[26] It has a weak affinity for plasminogen (K_m = 65 μM) in the absence of fibrin, but a much higher affinity when fibrin is present (K_m = 0.16 μM). Kinetic data support a mechanism in which t-PA and plasminogen adsorb to a fibrin clot in a sequential and ordered way, yielding a ternary complex.[21] Fibrin essentially increases the local plasminogen concentration, creating an additional interaction between t-PA and its substrate. Plasmin formed on the fibrin surface has both its lysine-binding sites and active sites occupied and is only slowly inactivated by α_2-antiplasmin. When t-PA is not bound to fibrin it circulates in human blood partly as free t-PA and partly as a complex with protease inhibitors. Low levels of t-PA in complex can be found in plasma of healthy volunteers and higher levels in pathologic plasma samples, like in patients after a myocardial infarction. The main inhibitor of t-PA is plasminogen activator inhibitor type-1 (PAI-1) and other inhibitors include C1-inhibitor, α_2-antiplasmin, α_2-macroglobulin and α_1-antitrypsin.[27] The liver exclusively eliminates t-PA from the blood.[28] t-PA has an initial half-life of 3.3 ± 0.4 minutes and a terminal half-life of 26 ± 12 minutes. It has been demonstrated in experiments in humans that liver blood flow is the rate limiting step in the clearance of t-PA.[29] The drug is not known to provoke immunologic or allergic reactions.

Pro-urokinase

The unglycosylated human single chain urokinase-type plasminogen activator (scu-PA) or pro-urokinase is an inactive single-chain precursor of urokinase which, following limited digestion with plasmin, is converted to fully active two-chain urokinase (tcu-PA) by hydrolysis of the Lys 158-Ile159 peptide

bond.[30,31] It is produced by genetically transformed E. coli.[20] Pro-urokinase is a true enzyme, as addition of a plasmin inhibitor (aprotinin or α_2-antiplasmin) abolishes the conversion of pro-urokinase to urokinase but not the activation of plasminogen to plasmin in purified systems. Scu-PA also activates plasminogen directly predominantly in the presence of fibrin. If fibrin is present plasminogen activation occurs, resulting in formation of fibrin associated plasmin. Therefore, scu-PA can be considered a fibrin specific thrombolytic substance. To what extent pro-urokinase is important for direct clot lysis or whether clot lysis mainly results from urokinase formation is not known. Scu-PA, like t-PA, is not known to induce immunologic or allergic reactions. It is also rapidly cleared from the plasma with a half-life of approximately 8 minutes[32,33] and it is assumed that this is accomplished for a large part by the liver.[34] It is likely that extraction in a single pass through the liver is so high that the blood flow to the liver becomes rate determining for its clearance.

Winner takes it all

After the introduction of the new thrombolytic drugs scu-PA and t-PA hopes of having found the ideal compound for treatment of acute myocardial infarction were high. The clot selectivity of these activators of fibrinolysis, especially of t-PA, appeared to be an important advantage. Clot lysis could be accomplished in doses that did not induce a systemic lytic state characterized by consumption of α_2-antiplasmin, depletion of plasminogen, fibrinogenolysis, increased fibrinogen degradation products (FDPs), and predisposition to systemic bleeding.[35,36] Definitive surgical treatment of high residual coronary stenosis could be initiated promptly after successful thrombolysis with the new agents in contrast to conventional activators because of their short biological half-life and their lack of induction of a systemic lytic state. Bleeding from arteriotomy sites could be less frequent after lysis induced with scu-PA or t-PA compared with streptokinase and other non-clot selective activators because of the anticoagulant effects of elevated FDPs and prolonged depletion of fibrinogen induced by the older agents. Early treatment of suspected infarction, offering the greatest opportunity for myocardial salvage, could perhaps be more justifiable with a clot-selective activator because risk associated with a systemic lytic state could be avoided. Furthermore, systemic administration of large doses seemed to be more practical with scu-PA and t-PA compared with streptokinase or urokinase without the need for intensive monitoring of the fibrinolytic system because of the clot selectivity.

However, it became clear that clot selectivity, even for t-PA, was relative. If high amounts of t-PA were present in the circulation, the formation of plasmin proceeded rapidly despite the relatively low affinity of circulating plasminogen. If a sufficient amount of plasmin was formed, α_2-antiplasmin was consumed, and free plasmin accumulated in the circulation, with a systemic lytic state as the end

result. However, the concentration of circulating t-PA could be limited by its short half-life and by the dose and the duration of infusion of t-PA. Clot selective dosage regimens would be those yielding a concentration of t-PA that was sufficient to lyse clots rapidly, but too low to form circulating plasmin in quantities that would allow the existence of a systemic lytic state. Therefore, after systemic administration of the 'correct' dose of t-PA, effective coronary thrombolysis should be possible without the occurrence of an increased bleeding tendency, assuming a relationship between the systemic lytic state and clinical bleeding. Clinical trials showed that with a variety of dose regimens t-PA indeed was an efficient thrombolytic drug. However, when the efficacy of t-PA was compared with the non-clot selective activators, the expected superiority of tissue-type plasminogen activator could not be clearly shown.

Clinical trials - efforts to find the correct dose

In 1984 observations indicated that clot selective coronary thrombolysis could be induced in patients with evolving myocardial infarction by means of t-PA.[37] Seven patients were treated with intracoronary and/or intravenous administration of t-PA. Two patients received a single intravenous treatment of $1.2x10^6$ IU in 30 minutes and $1.4x10^6$ IU in 35 minutes. Lysis was complete after 22 and 19 minutes respectively, but in the first patient reocclusion and reinfarction occurred 18 days after t-PA infusion.

The European cooperative study group for recombinant tissue-type plasminogen activator (rt-PA) reported in 1985 a comparison of intravenous rt-PA with intravenous streptokinase (1.5 million IU in 60 minutes) in acute myocardial infarction.[38] A dose of 0.75 mg rt-PA/kg (approximately 55 mg) was administered over 90 minutes. No significant difference in patency of the infarct-related vessel was found between the two thrombolytic drugs. Bleeding episodes and other complications were less common in the rt-PA patients and the activation of the fibrinolytic system was far less pronounced in this group.

In 1986 a study was performed that concluded that the efficacy of rt-PA for coronary thrombolysis was dose-dependent. Infusion rates of 5 μg/kg/min or more for 90 minutes accomplished reperfusion in more than 80% of the patients. However, the frequency of occurrence of residual intraluminal thrombus was significantly lower with an infusion rate of 7 μg/kg/min for 90 minutes. When this initial rt-PA infusion was followed by an intravenous maintenance infusion of 2 μg/kg/min for 4 hours, only modest additional fibrinogen breakdown was found.[39]

In the TIMI-2 trial 3262 patients were treated with rt-PA.[40] The first 520 patients received 150 mg rt-PA in 6 hours (bolus of 9 mg followed by 90 mg in the first hour, 20 mg in the second hour and 10 mg in each of the next 4 hours). Because of an unacceptably high rate of intracranial haemorrhage, the dose was subsequently reduced to 100 mg in the remaining 2742 patients (bolus of 6 mg

followed by 54 mg in the first hour, 20 mg in the second hour and 5 mg in each of the next four hours.

The third international study of infarct survival (ISIS-3) reported in 1992 that, with a rt-PA dose administered by weight of 0.6 MU/kg (± 100 mg) infused over 4 hours, 5 per 1000 fewer reinfarctions and 4 per 1000 more strokes were found with rt-PA compared to streptokinase treatment.[41]

Neither the GISSI-2 international trial[42] nor the ISIS-3 trial found a difference in associated mortality between the use of streptokinase and the use of t-PA. However, the GUSTO investigators reported in 1993 that their findings indicated that accelerated t-PA given with intravenous heparin provided a survival benefit over previous standard thrombolytic regimens.[43] In this study the dose was a bolus of 15 mg rt-PA followed by an infusion of 0.75 mg/kg over a 30 minute period, not to exceed 50 mg, and 0.5 mg/kg, up to 35 mg, over the next 60

Table I. Dose regimens that were administered in the different clinical trials during more than 10 years of thrombolytic treatment with rt-PA.

Study	Year	Patients	Doses of rt-PA	Time (min)
- van der Werf et al.	1984	1	$1.0x10^6$ IU	45
		2	$6.0x10^5$ IU	30
		2	$1.2x10^6$ IU	30
		1	$1.4x10^6$ IU	35
- European Cooperative Study Group	1985	64	55 mg*	90
- TIMI-1	1985	112	80 mg	180**
- Garabedian et al.	1986	6	30 mg*	90
		7	32 mg*	90
		9	42 mg*	90
		7	49 mg*	90
		16	39 mg*	60
- TIMI-2	1989	520	150 mg	360†
		2742	100 mg	360†
- GISSI-2	1990	6182	100 mg	180†
- ISIS-3	1992	13746	100 mg*	240†
- TIMI-3	1993	199	100 mg	180†
- GUSTO	1993	10396	100 mg*	90†
- TIMI-5	1994	252	100 mg*	90†

* = Dose was corrected for body weight.
** = Administration of a 'lytic' dose immediately followed by a 'maintenance' infusion.
† = Administration of a 'lytic' dose preceded by a bolus injection and followed by a 'maintenance' infusion.

minutes. Compared with streptokinase a significant excess of haemorrhagic strokes for accelerated t-PA was found.

In 1990 convincing evidence was presented that plasmin bound to fibrin continuous to be active long after exogenous t-PA was cleared from the circulation. It was concluded that the data seemed to indicate that t-PA infused as a bolus dose is at least as effective, and probably more so than t-PA infused over a longer period.[44] The major advantage of a bolus administration seemed to be decreased bleeding tendency. Moreover, the dose regimens that included a slower 'maintenance' rate, designed to prevent reocclusion, became questionable since the only controlled study of this regimen failed to show any benefits from this maintenance infusion.[45]

Variability

Dose regimen versus variability

For more than a decade investigators have tried to adjust the dose regimen of rt-PA to accomplish an optimal balance between the benefits and risks of rt-PA treatment (Table I). Compared to other thrombolytic drugs, a higher percentage of reperfusion, a lower mortality rate, a lower rate of haemorrhagic strokes, fewer reports of allergy and of non-cerebral bleeds and a low rate of reinfarctions had to be achieved. Although a formal agreement on the correct dose has not yet been established, an optimal dose regimen must ultimately be determined. However, even then, as was seen in all large clinical studies, there will be patients in whom reperfusion of the coronary vessel will not be successful. In other patients serious adverse events, like a haemorrhagic stroke, will occur.

This does not necessarily indicate that these patients received an incorrect dose dose, but in these patients inappropriate plasma concentrations may have ensued. In many studies large interindividual differences in steady state t-PA concentrations of patients with acute myocardial infarction were found after infusion of equal doses of rt-PA.[39,46,47] However, in healthy volunteers the variability in plasma concentrations was not so large.[48] The interindividual variability in plasma concentrations between patients should be taken into account when comparing dosage regimens. If the variability is large, a substantial proportion of patients with concentrations outside the therapeutic range may arise. In patients with low thrombolytic plasma concentrations reperfusion of the occluded coronary vessel would not occur or, if reperfusion did take place, early reocclusion would be highly probable. With plasma concentrations that are too high, chances of developing major bleeding complications would almost certainly increase (Fig. 2). These events could explain the sometimes disappointing results after rt-PA administration when the drug is compared to other thrombolytic treatments like streptokinase. Therefore,

it is important to investigate factors that could have an influence on the variability. Administration of thrombolytic treatment by body weight, the role of liver blood flow on the concentrations of rapidly cleared drugs, the capacity of several inhibitors to bind plasminogen activators in plasma or the role of the endothelium in releasing or binding thrombolytic agents all could have effects on variability of steady state plasma levels.

Dosing by weight

The dosage regimen for rt-PA that is currently used includes an initial bolus injection, followed by a high 'lytic' dose infusion and a subsequent infusion at a slower 'maintenance' rate. This regimen does not take into consideration the body weight of the patients that are involved. A study was performed where in phase I patients received treatment without weight adjustment and in phase II the 'normal' dose was adjusted for body weight to the nearest 5 kg and delivered in a lytic dose of 1.2 mg/kg over 60 minutes, preceded by a bolus 0.12 mg/kg over 1 minute and immediately followed by a maintenance infusion of 0.69 mg/kg over 3 hours.[49] The results showed that in both groups a large variability in plasma concentrations was still present. However, the average observed steady state lytic activity concentrations in phase II were significantly lower than in phase I. Therefore, dosing by weight and the consequent reduction in variability of plasma concentrations should theoretically improve efficacy, with a reduced chance of side effects.

Liver blood flow and plasma concentrations of t-PA

Assuming complete bioavailability, steady state plasma concentrations (C_{ss}) of a drug will be achieved when the rate of drug elimination (Clearance = CL) equals the rate of drug administration (IR = infusion rate). Clearance is defined as the volume of body fluid that is cleared from the drug per unit of time. Therefore, it does not indicate how much drug is being removed. For most drugs clearance is not dependent on the concentration of the drug. This means that the system for elimination can not be saturated and a constant fraction of the amount of drug in the body is eliminated per unit of time. If a single organ is responsible for the clearance from the body, the elimination rate (R) by this organ is determined by $R = (Q*C_A) - (Q*C_V)$, with Q the flow through the organ and C_A and C_V the arterial and venous concentration. The value of the clearance of a drug is defined by the equation $CL = Q * ((C_A - C_V)/C_A))$ and thus determined by the blood flow through the organ of elimination (Q) and the efficacy of the elimination or extraction ratio in this organ $(C_A - C_V)/C_A)$. If an organ can eliminate almost all of the drug with a single pass of the blood through that organ ($C_V \rightarrow 0$), then the drug is called a 'high clearance drug'. If the organ only

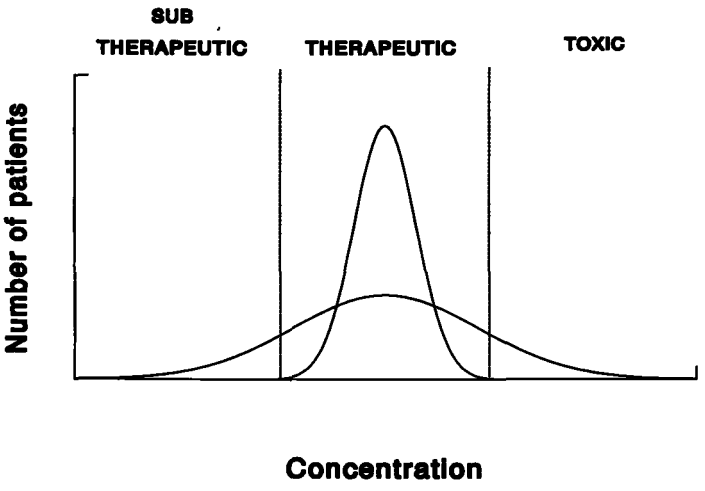

Figure 2. Assuming a therapeutic range, a large variability in plasma concentrations (broad curve) will increase the number of patients with subtherapeutic ranges and with toxic concentrations. Reducing the variability (narrow curve) will increase the efficacy and decrease the side effects in a trial.

eliminates a small fraction with one pass, the drug is called a 'low clearance drug'. The elimination of 'high clearance drugs' is dependent on the blood flow through the organ of elimination, whereas the clearance of 'low clearance drugs' is dependent on the extraction ratio (Fig. 3). Substances with a high extraction ratio can be used as a measure of the blood flow through the organ where the substance is cleared. Although the steady state concentration of a high clearance substance is determined by the organ blood flow, changes in organ blood flow are only reflected in the plasma concentrations if the duration of the change in flow is long enough to reach a new steady state (approximately 4 to 5 half-lives).

Both t-PA[28] and scu-PA are drugs that are considered to be efficiently removed from the blood by hepatic processes through receptor-mediated mechanisms. For scu-PA and two chain urokinase type plasminogen activator evidence has been provided that the low density lipoprotein receptor-related protein/α_2-macroglobulin receptor is responsible for clearance of these molecules from the circulation.[34] A specific, not easily saturated, high-affinity binding site for t-PA was identified after investigation of membranes prepared from human liver.[50] The concentration of the drugs leaving the liver will be low and it is postulated that the clearance from blood will become limited by hepatic blood flow. The influence of reduced liver blood flow on the kinetics of t-PA has been investigated by de Boer et al.[29] A reduction of liver blood flow was assessed by performing an exercise test. With an average reduction in flow of 57% an

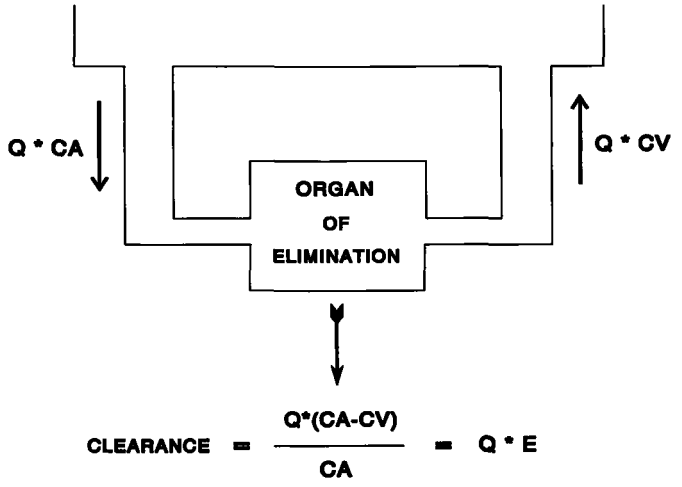

Figure 3. Schematic representation of the clearance of a substance by an organ of elimination. Q = Flow through the organ, C_A = Arterial concentration, C_V = Venous concentration, E = Extraction ratio.

increase in t-PA activity by 119% and t-PA antigen by 91% was observed. As theory predicts[51] an increase in liver blood flow does not affect plasma concentrations as much as a decrease (Fig. 4).

Myocardial infarction could be the cause of a decreased liver blood flow by impairment of cardiac output. The patients who received a standard dose of the thrombolytic drug but who all had more or less different levels of congestive heart failure could have very different steady state concentrations as a result of the variation in clearance. This was already shown in 1978 for the widely used intravenous antiarrythmic agent lidocaine.[52] Lidocaine has a high extraction ratio and hepatic blood flow should have an important influence on the removal rate and thus on the plasma levels of lidocaine. The importance of hepatic blood flow on lidocaine kinetics was studied by comparing estimations of hepatic plasma flow to lidocaine clearance in patients with various degrees of congestive heart failure. The results demonstrated that patients with congestive heart failure had significantly higher steady state lidocaine levels and that the regression line relating lidocaine clearance and liver blood flow was linear. If a similar relationship between liver blood flow and scu-PA and t-PA concentrations in patients with myocardial infarction could be determined, the large variation in steady state plasma concentrations of both drugs could at least partly be explained.

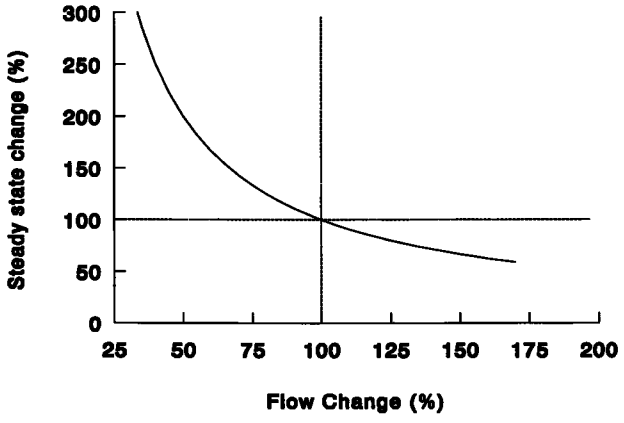

Figure 4. Correlation between liver blood flow and steady state concentration of a 'high clearance drug', assuming that change in flow is constant over a period that is long enough (four to five half-lives) for the 'high clearance drug' to reach a new steady state.

Inhibitors of plasminogen activators

Variability in plasma concentrations of plasminogen activators may be due to differences in clearance, but also due to variable inactivation in the circulation. The activity of the physiological concentrations of plasminogen activators in human plasma is regulated by protease inhibitors. Three immunologically distinct plasminogen activator inhibitors (PAI) can be identified, but the endothelial cell type (PAI-1) appears to be the main physiological inhibitor in normal plasma.[53,54] The remaining t-PA or u-PA is only partly present in a free form because other inhibitors, like C1-inhibitor, α_2-antiplasmin, α_2-macroglobulin and α_1-antitrypsin, also can play a role in the regulation of plasminogen activator activity. However, the reaction rate of these inhibitors is slow and therefore PAI-1, with its rapid reaction rate, is the dominating element that controls the level of free t-PA and u-PA in normal human blood.[27] When a plasminogen activator is given in pharmacologic doses to patients with acute myocardial infarction, the fast acting inhibitors are probably saturated. The fact that t-PA activity is cleared more rapidly during thrombolytic therapy cannot be solely explained by the inhibition of PAI-1.[49,55] It has been observed that in patients, endogenous t-PA circulated predominantly as a complex with PAI-1, while during infusions t-PA not only circulated as free t-PA but also in complexes with PAI-1, α_2-antiplasmin and C1-inhibitor.[56] It was concluded that infused t-PA initially saturates PAI-1. The role of α_2-antiplasmin and C1-inhibitor in binding to t-PA could then become

more important.

The serine proteinase inhibitor α_2-antiplasmin is an extremely efficient, fast reacting inhibitor of plasmin. The rate of fibrinolysis is controlled by the rate of inactivation of generated plasmin. Plasmin in a free solution has free lysine binding sites. In this situation the reaction with α_2-antiplasmin is much faster compared to plasmin with saturated lysine binding sites. Since the lysine binding sites on plasmin are involved in interactions with fibrin, this plasmin is spared but plasmin that has diffused away from the clot will be rapidly inhibited, hereby allowing efficient fibrinolysis.[57] As mentioned above, excess t-PA forms complexes with low affinity inhibitors like α_2-antiplasmin and the single chain glycoprotein C1-inhibitor.[58] If a high dose of t-PA is infused over a long period, the concentrations of t-PA/α_2-antiplasmin will decrease because of a depletion of α_2-antiplasmin associated with generation of plasmin in the circulation by administered t-PA.[36]

If after infusion of therapeutic doses of rt-PA in patients with myocardial infarction the contribution of t-PA in complex, compared to not bound t-PA, is substantial, part of the interindividual variation in steady state concentrations could be explained. To investigate this possibility a quantification of the complex formation between t-PA and C1-inhibitor and α_2-antiplasmin should be made. Assays must be developed for precise measurements of the complexes. Only then, differences in clearance of the various t-PA/inhibitor complexes can be investigated. If the half life of t-PA/C1-inhibitor complex and t-PA/α_2-antiplasmin complex would be different from the half life of free t-PA and consequently t-PA in complex would be cleared differently, a variation in steady state concentrations of t-PA could exist, depending on the amount of complex formation per patient.

Role of the endothelium

Plasminogen activators play an important role in the lysis of the fibrin and the angiogenesis. Both t-PA and u-PA and the plasminogen activator inhibitor type 1 are synthesized by endothelial cells. Under normal physiological circumstances fibrinolysis is predominantly regulated by the production and release of t-PA. For local proteolysis of the endothelial extracellular matrix, which allows generation and development of new capillaries in case of tissue damage, u-PA is the regulating factor. The plasma levels of the plasminogen activators can be increased by several stimuli like mechanical vascular occlusion[59], exercise[60,61] or by agents like bradykinin and platelet activating factor. To regulate the functions of t-PA and u-PA there are specific binding sites on the endothelial cells.[62,63]

In patients in the acute phase of myocardial infarction a procoagulant state exists with a shift in the balance within the fibrinolytic system of profibrinolytic factors like t-PA and antifibrinolytic factors like PAI-1.[64] The endothelium synthesizes and/or releases PAI-1 at a faster rate in the blood than t-PA,

resulting in increased thrombus formation. Different levels of dysregulation of the endothelium could be the cause in the diversity in the development of a thrombus and also in the success of lysis. Underlying factors, like smoking, hypercholesterolemia, homocystinaemia, diabetes mellitus or hypertension could alter the function of the endothelium and thereby influence the severity of thrombus formation leading to an acute myocardial infarction. The same factors could be of importance when these patients are given thrombolytic treatment. The working mechanism of the plasminogen activator could be distorted at receptor level, thereby providing different results of reperfusion with in principle similar levels of thrombolytic drug. It is not likely that a large part of the variability in steady state levels of u-PA or t-PA will be caused by the variety in overall endothelial binding between the patients. To reach therapeutic levels high doses are necessary and this large quantity is perhaps relatively indifferent to a variation in binding places. However, changes in the receptor uptake in the liver could prove important by substantially changing the clearance of u-PA or t-PA.

A study was performed in which a low dose intravenous infusion of 80 μg rt-PA was administered over 16 minutes.[60] A delay in appearance of rt-PA in the venous circulation was detected and the concentration profile displayed a concave profile in the first minutes after starting the infusion. This was an indication that a portion of the rt-PA did not reach the circulation, suggesting another unidentified compartment that might involve binding to the endothelium. It appeared that this process was saturable and the possibility was raised that quantification of the amount of endothelium involved was relevant for t-PA physiology and pathophysiology.

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CHAPTER 2

OBJECTIVES AND METHODS OF THE STUDIES DESCRIBED IN THIS THESIS

Effective blood concentrations of t-PA and u-PA are the end result of clearance, binding to components in the blood and binding to the endothelium. The removal from the body of these 'high clearance drugs' is dependent on their perfusion through the organ of elimination. Indeed, it has been demonstrated in healthy volunteers that decreased liver perfusion has a direct influence on the blood concentrations of t-PA.[1]

The first two studies in this thesis were performed to investigate the influence of changes in liver blood flow on the pharmacokinetics and pharmacodynamics of saruplase (a single chain urokinase-type plasminogen activator) and alteplase (a tissue-type plasminogen activator). These studies were performed in healthy volunteers to eliminate the influence of concurrent vascular disease and concomitant medication. Increased liver blood flow was accomplished by administration of a standard lipid/protein rich meal. Fat provides the greatest individual stimulus to human intestinal blood flow[2] and a high-protein meal increase hepatic blood flow.[3,4] A decrease in liver blood flow was obtained by a standardized exercise test. Indocyanine green was used to estimate liver blood flow. This tricarbocyanine dye is rapidly distributed in the circulating plasma and does not reach extravascular compartments. It has a short half-life and is exclusively removed by the liver without biotransformation[5,6] and does not undergo enterohepatic circulation. The hepatic extraction ratio in man is high[7] ($\geq$ 0.7) and therefore its clearance is related to liver blood flow. Liver blood flow was measured independently by echography of the portal venous system.

The effect of changes in liver perfusion on blood concentrations of t-PA and scu-PA is important, because liver blood flow between patients with acute

myocardial infarction can vary by a factor ten, dependent on the haemodynamic situation of the patient. This could result in very high thrombolytic plasma concentrations after administration of a standard dose and must not be seen separately from the higher incidence of haemorrhagic strokes after t-PA administration compared to streptokinase.[8,9] Therefore, two studies were performed to investigate the relationship between liver blood flow and thrombolytic blood concentrations in patients with acute myocardial infarction. After administration of a bolus injection of 20 mg saruplase followed by a one-hour infusion of 60 mg saruplase and the concurrent infusion of indocyanine green, a comparison was made between the clearance values of saruplase and those of indocyanine green. A similar study was performed after the administration of standard thrombolytic treatment with rt-PA (a bolus injection of 10 mg rt-PA followed by a continuous infusion of 50 mg over 60 minutes and 40 mg over the next 120 minutes).

There are several inhibitors of t-PA that circulate in the human blood. Plasminogen activator inhibitor type-1 (PAI-1) reacts rapidly with t-PA,[10] but other more slowly acting inhibitors might become more important during thrombolytic treatment because PAI-1 will be depleted because of the high levels of t-PA. These complexes have not been quantified nor has the half-life of these complexes been determined in blood. If clearance of these complexes would be significantly different from clearance of not bound t-PA, an additional explanation would be provided for the extensive variation in thrombolytic plasma concentrations in acute myocardial infarct patients. Therefore, the role of two important slow acting inhibitors C1-inhibitor and α_2-antiplasmin was investigated specifically. An enzyme-immuno assay was developed to measure tissue-type plasminogen activator/C1-inhibitor complexes. In vivo formation of this complex was studied in healthy volunteers in normal plasma to determine the amount of complex formation and to define the pharmacokinetic properties. t-PA/C1-inhibitor complex formation was also investigated in plasma of healthy volunteers before and after venous occlusion, after an exercise test, after administration of desmopressin (DDAVP),[11] after administration of an infusion of rt-PA (18 mg/120 min) during which time an exercise test was performed[1] and in plasma and peritoneal fluid from patients suffering from pelvic inflammatory disease. For a second study inhibition of t-PA by purified α_2-antiplasmin was investigated with a newly developed sandwich immunoassay. Complex formation of both t-PA/C1-inhibitor complex and t-PA/α_2-antiplasmin was studied by separating free from bound t-PA using gel filtration. In vivo complex formation was studied in patients receiving thrombolytic treatment to determine if levels of complexed t-PA could be an explanation for the interindividual variability in steady state blood levels of t-PA.

The study of de Boer et al.[12] gave an indication of presystemic adsorption of t-PA in an unidentified compartment during infusion of a low dose. This unidentified compartment could be the endothelium and if so, quantification of

the amount of endothelium involved could be relevant as a measure of endothelial dysfunction in various patient groups. However, the deviations that were found in the study of de Boer could have been caused by another mechanism.

We investigated if adsorption of rt-PA to the syringe or infusion line had occurred. In further experiments the influence of the duration of time between the preparation of the syringe and infusion line and the start of the infusion was studied. The binding capacity of t-PA to blood cells was also investigated. Finally, the in vivo experiment of de Boer was repeated with the administration of two low dose infusions of rt-PA.

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Section II

PHARMACOLOGICAL STUDIES IN HEALTHY VOLUNTEERS

CHAPTER 3

EFFECTS OF CHANGING LIVER BLOOD FLOW BY EXERCISE AND FOOD ON KINETICS AND DYNAMICS OF SARUPLASE

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Summary

Objective: *To investigate the influence of changes in liver blood flow on the pharmacokinetics and pharmacodynamics of single-chain unglycosylated urokinase-type plasminogen activator.*

Methods: *This open, randomized, crossover trial was carried out in a clinical research unit. Infusions of 37.5 mg saruplase and 90 mg indocyanine green were administered over 150 minutes. After 60 minutes the subjects consumed a standardized meal to increase liver blood flow or performed an exercise test (20 minutes) to decrease liver blood flow. Indocyanine green concentrations, total urokinase-type plasminogen activator (u-PA) antigen, two-chain u-PA activity, fibrinogen, total degradation products, α_2-antiplasmin, and factor XII-dependent fibrinolytic activity were measured. Blood flow was measured after food intake in a portal vein branch with Doppler echography.*

Results: *The weighted average indocyanine green concentration after exercise was increased by 29% compared with baseline (steady state concentration) values*

(95% confidence intervals [CI]:+6%,+56%). After food, the concentration was 27% lower compared with baseline values (95% CI:-35%,-19%) and portal vein flow was increased by a maximum of 103% (95% CI:+71%,+136%). Average maximal concentrations of u-PA antigen after exercise were increased by 130 ng/ml compared with baseline concentrations (95% CI:+65 ng/ml, +195 ng/ml) and, unexpectedly, 156 ng/ml higher after food (95% CI: +59 ng/ml,+253 ng/ml). Although not significant, an increase in average u-PA antigen concentration compared with baseline values was detected after both exercise (7%) and food (13%). This tendency toward a larger effect after food compared with the effect after exercise was reflected by minor changes in the pharmacodynamics.

***Conclusions:** u-PA plasma concentrations were increased by reduced liver blood flow induced by exercise. Food intake produced an unexpected increase in u-PA concentrations despite increases in liver blood flow.*

Introduction

Plasmin is an enzyme that dissolves the fibrin of a blood clot. Thrombolytic drugs are activators of the conversion of the inactive proenzyme plasminogen to the proteolytic enzyme plasmin. This property has been applied clinically in patients with myocardial infarction,[1,2] deep vein thrombosis[3] or pulmonary embolism.[4] Single-chain urokinase-type plasminogen activator or pro-urokinase is a new thrombolytic agent.[5,6] It is a single-chain glycoprotein containing 411 amino acids that can be converted to active two-chain urokinase.[7] Activation of plasminogen is not only accomplished by urokinase but, to a lesser extent, by single-chain u-PA,[8] and the activation is catalyzed by fibrin. The recombinant unglycosylated molecule expressed in *Escherichia* coli (rscu-PA; INN, saruplase) has been solely investigated for the treatment of acute myocardial infarction.[9]

Recombinant single-chain u-PA is cleared very rapidly from the plasma with a half-life of 7.9 minutes,[10] and it is assumed that this is accomplished in large part by the liver.[11,12] Extensive variability in liver blood flow has been shown in patients with heart failure.[13,14] High concentrations of the thrombolytic drugs could cause an increased bleeding risk. Low concentrations could possibly result in insufficient thrombolysis. Therefore it is important to investigate the influence of liver blood flow on the pharmacokinetics of saruplase. Moderate to strenuous exercise reduces liver blood flow and induces a diminished elimination of high-clearance drugs.[15] An increase in blood flow and the elimination of high-clearance drugs can be obtained by food.[16] Indocyanine green clearance is widely used as a marker of the hepatic blood flow.[17] It is entirely cleared by the liver,[18] and its hepatic extraction after an intravenous bolus injection has been shown to average 0.7 in normal subjects, with a limited intersubject variability.[19] Clearance techniques can be relatively insensitive to increases in liver blood flow.[20] Therefore, portal blood flow after the intake of food was also measured

with echo-Doppler.

This article reports an investigation of the influence of decreased liver blood flow, induced by exercise, and increased liver blood flow, induced by food intake, on the pharmacokinetics of saruplase and the effects on selected parameters of the coagulation and fibrinolytic system.

Materials and methods

Patients

A group of 10 nonsmoking healthy male volunteers (age range, 21 to 29 years; weight range, 62 to 81 kg) participated in this open randomized crossover study after informed consent was obtained. None of the subjects had a condition known to be associated with an increased bleeding risk. The volunteers were in good health as judged by medical history, physical examination, 12-lead electrocardiogram, hematology, blood chemistry, activated partial thromboplastin time, thrombin time, prothrombin time, bleeding time, fibrinogen level, and urinanalysis. None of the subjects received concomitant medication or recent medical treatment. Subjects had not participated in a clinical trial during the last 6 months before the start of the study, had not donated blood within 3 months of the start of the study, and did not show evidence of a recent trauma (with hematoma) or surgery in the 4 weeks preceding the study. Subjects were studied after abstaining from alcohol for 48 hours. Caffeine-containing beverages and alcohol were not allowed during the study. The protocol of the study was approved by the Ethics Committee of Leiden University Hospital.

Treatments

A total amount of 90 mg indocyanine green (0.6 mg/min) was given by intravenous infusion (100 mg in a 40 ml aqueous solvent) and a total amount of 37.5 mg saruplase (0.25 mg/min) was given concurrently (in 48 ml) by intravenous infusion over a time period of 150 minutes. Saruplase was supplied by Grünenthal GmbH, Aachen, Germany, and indocyanine green ampoules (Cardiogreen, Hynson, Westcott & Dunning, Baltimore, Md.) by the Leiden University Hospital Pharmacy. All the medication was administered with use of syringe infusion pumps (Harvard Apparatus syringe infusion pump, model 22, South Natick, Mass.). An interval of 2 weeks separated the two study periods. Saruplase was not administered without intervention (as control administration) to limit the amount of blood taken and the exposure of subjects to saruplase.

Subjects arrived at the study center after an overnight fast. The volunteers remained sitting during the entire experiment. Two intravenous cannulas for administration of medication were inserted, and a third cannula was inserted in

the contralateral arm for blood collection. Premedication blood and urine samples were collected before the 150-minute infusion of indocyanine green and saruplase started. Before administration the volunteers rested for 90 minutes. Sixty minutes after the infusions were started, the subjects performed an exercise test over a period of 20 minutes or consumed a lipid- and protein-rich meal in 10 minutes. The standardized exercise test was performed on an electromagnetically braked bicycle ergometer (Lode, Groningen, The Netherlands). Before the study the volunteers were tested to determine the individualized load for the study by starting the load at 50 W/min and increasing it by 25 W/min until a heart rate of 150 beats/min was reached. On the study days, 85% of this load was applied. The meal consisted of 600 kcal (25% energy from protein, 51% energy from lipid, 24% energy from carbohydrate). It is reported that fat provides the greatest individual stimulus to human intestinal blood flow,[21] and several investigators found that a high-protein meal increases hepatic blood flow.[22,23] After 150 minutes the two infusions were stopped; 12 hours after start of the infusions the subjects returned home, provided that the fibrinogen level was in the normal range.

Doppler echographic measurements

Measurements were made with an echograph equipped with pulsed Doppler facilities (Toshiba model SS250A; Toshiba Corporation, Tokyo, Japan) with a 3.75 MHz transducer. The hepatic portal venous system was visualized by intercostal approach. Measurements were made of the diameter of the main portal vein and an intrahepatic branch. From the latter vessel the (spatial- and time- averaged) mean blood flow velocity (V_{mean}) was also measured from the Doppler spectrum with use of the apparatus' own software. This approach was chosen because it allows measurement of blood flow velocity with small angles of insonation. The mean blood flow in the portal vein branch was calculated by the formula $Q = CSA * V_{mean}$, in which CSA is the cross-sectional area of the vessel, calculated from the diameter and assuming a circular geometry. Measurements were made in duplicate and in the same phase of respiration.

Blood sampling

Blood was collected after the contents of the cannula were discarded. A total amount of approximately 670 ml was collected. On a study day with the exercise test, samples for determination of saruplase (3 ml in 0.1 volume of 3.8% citrate with aprotinin and benzamidine) and indocyanine green (3 ml in tubes that contained lithium heparin) were taken shortly before and at 20, 30, 40, 48, 53, 56, 59, 64, 67, 70, 73, 76, 79, 81, 84, 87, 90, 95, 100, 110, 120, 130, 140, 151, 155, 160, 165, 175, 190, 210, and 240 minutes after drug administration.

When a meal was consumed, samples were taken shortly before and at 20, 30, 40, 48, 53, 56, 59, 64, 75, 80, 84, 90, 95, 100, 110, 115, 120, 125, 130, 135, 140, 145, 149, 151, 155, 160, 165, 175, 190, 210, and 240 minutes after drug administration. On both occasions blood for fibrinogen, α_2-antiplasmin, and total degradation products (3 ml of blood with prostaglandin E_2, theophylline and Glu-Gly-Arg-chloromethyl-ketone) was taken before infusion and at 56, 120, 175, 270, and 720 minutes. For factor XII-dependent fibrinolytic activity, blood was drawn (4.5 ml in tubes containing sodium citrate) before and 720 minutes after the start of the infusion. After centrifugation the plasma was separated and stored until analysis at -40°C for the indocyanine green assay and at -70°C for the other assays.

Assays

Plasma levels of indocyanine green were determined by HPLC according to Rappaport and Thiessen[24] in a slightly modified form, with use of diazepam as internal standard. The assay had a detection limit of 0.1 μg/ml. Total u-PA antigen (saruplase and degradation products) was determined by means of an ELISA technique (detection limit of 2 ng/ml).[25] Pre-treatment values were directly subtracted from subsequent time points. An immunoactivity assay with Glp-Gly-Arg-pNA substrate (S-2444, Kabi Diagnostica, Mölmdal, Sweden) was used to measure the two-chain u-PA activity (detection limit of 3.4 ng/ml).[25] α_2-Antiplasmin was determined by the immediate plasmin inhibition test.[26] The detection limit of the assay was 12.5% of the normal plasma concentration. Fibrinogen was measured according to Clauss (detection limit of 0.5 g/L)[27] and total degradation products were measured according to Koopman et al.[28] (detection limit of 5 ng/ml). The factor XII-dependent fibrinolytic activity was assayed in the dextran sulphate euglobulin fraction of plasma in the presence of sodium flufenamate and specific antibody neutralizing activities of both t-PA and urokinase was added.[29,30] The detection limit for the method was 1.5 BAU/ml (BAU, blood activator units).

Data processing

Pharmacokinetic parameters for total u-PA antigen were calculated by means of a standard two-compartment open pharmacokinetic model (Siphar software package; Simed, Créteil, France). The u-PA antigen concentrations from the beginning of the intervention until the last sample before the end of the infusion were not taken into consideration for estimation of the kinetic parameters. The area under the curve (AUC), clearance (CL), half-life of the first exponential phase ($t_{½}\lambda_1$), half-life of the second exponential phase ($t_{½}\lambda_2$), and volume of distribution at steady state (V_{SS}) were computed with use of the fitted models.

Because earlier pharmacokinetic studies in healthy volunteers indicate steady state conditions after 50 minutes of continuous infusion, baseline values were calculated from the average of the last three u-PA or indocyanine green concentrations before the exercise test or food consumption was started (53, 56, and 59 minutes). All data were log-transformed to compensate for their supposed log-normal distribution. The averaged maximal values and weighted average concentration (AUC divided by the time span over which the AUC was calculated) of u-PA antigen were compared with baseline values. The weighted average concentrations of indocyanine green were also compared with baseline values. Comparisons of the weighted average concentrations of the pharmacodynamic parameters were performed to detect differences between treatments. A decrease in fibrinogen on each study day was tested by comparison of the pre-medication value with normalized AUC over 720 minutes. Values were compared with paired *t* tests and completed with 95% confidence intervals (95% CI). All calculations were carried out using SPSS/PC+ version 4.0.1 (SPSS Inc., Chicago, Ill.) computer software. For analysis of the Doppler echographic measurements, presence of a time effect was assessed by use of the statistical software module BMDP5V (BMDP/Dynamic 1990 version, BMDP Statistical Software, Inc., Los Angeles, Calif.). In case of significant time effects, paired *t* tests with the premeal value were performed to identify time points with significant differences.

Results

No relevant adverse events were observed. The mean indocyanine green plasma concentration-time profile is presented in Fig. 1. During a continuous infusion of saruplase and indocyanine green, exercise increased mean (±SD) indocyanine green plasma levels from 1.0 ± 0.3 μg/ml at baseline to 2.3 ± 0.6 μg/ml at its peak. The increase in indocyanine green concentrations from baseline to the end of the exercise was 135% (95% CI:+99%,+191%). The weighted average concentration after exercise was 29% higher compared with baseline (95% CI:+6%,+56%). An expected reduction in indocyanine green concentrations was observed after the standardized meal with a reduction in weighted average concentrations of 27% compared with baseline (95% CI:-35%,-19%). Adequate measurements of the portal vein branch could be made in all subjects. Visualization and accurate measurement of the main portal vein diameter was possible in eight subjects. The diameter of the main portal vein showed a maximal increase of 1.34 mm (95% CI:+0.74 mm,+1.93 mm) 80 minutes after food intake, corresponding to a 13% increase. The diameter of the portal vein branch increased maximally by 0.81 mm (95% CI:+0.40 mm,+1.23 mm), that is, 11% from baseline 60 minutes after food intake. Mean blood flow velocity increased maximally by 69% (95% CI:+47%,+128%). The increase in flow through the portal vein branch (Fig. 2) which started soon after the meal was

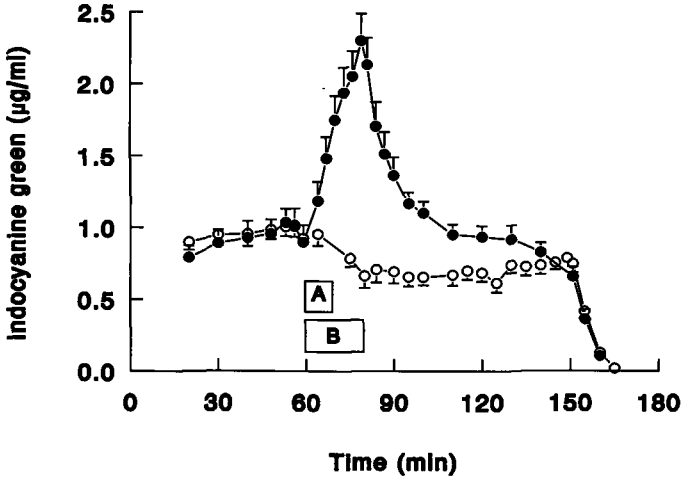

Figure 1. Mean ± SEM (N=10) plasma concentration-time profiles of indocyanine green (ICG) after an infusion of 90 mg ICG over 150 minutes with the consumption of a standard meal (-○-) in 10 minutes (A) or an exercise test (-●-) over 20 minutes (B) after 60 minutes. Both experimental interventions were carried out in the same subjects with a 2-week interval.

taken, reached a maximal change of 103% (95% CI:+71%,+136%) at 60 minutes and remained elevated over the entire duration of the experiment.

Pharmacokinetic parameters for total u-PA antigen were calculated with use of a standard two-compartment open pharmacokinetic model (Table I). The mean plasma concentration-time profile of total u-PA antigen is presented in Fig. 3. Total u-PA antigen reached a steady state before the exercise test or the standardized meal started. After exercise the averaged maximal concentration of 393 ± 113 ng/ml at 80 minutes was significantly higher than baseline concentrations of 263 ± 67 ng/ml (95% CI of the difference:+65 ng/ml,+195 ng/ml). After the standardized meal the average maximal concentration of 448 ± 141 ng/ml at 86 minutes was also significantly higher than baseline concentration of 292 ± 54 ng/ml (95% CI:+59 ng/ml,+253 ng/ml). Although not significant, an increase in weighted average concentrations could be detected with both the exercise (7%; 95% CI:-6%,+22%) and the standardized meal (13%; 95% CI: -5%,+34%) when compared with baseline. The concentration-time profile of two- chain u-PA activity appeared to show a similar response to exercise or the standardized meal as total u-PA antigen. However, interpretation of the increases could be realized only if steady state concentrations were reached before the interventions were made, and this was not clearly shown. Therefore comparisons of AUC values after exercise or food with a baseline AUC were not performed.

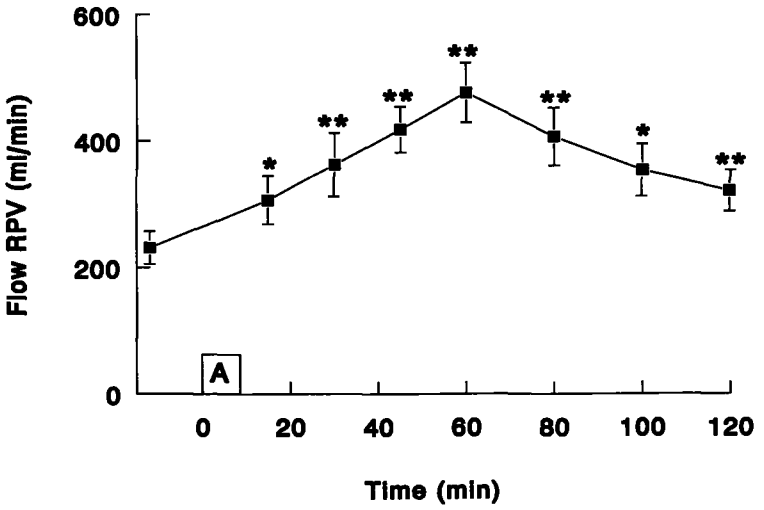

Figure 2. Time course of mean ± SEM blood flow in a portal vein branch (-■-) after an infusion of 37.5 mg saruplase over 150 minutes with after 60 minutes the consumption of a standard meal (A). Significant changes in flow compared to pretreatment values (average of two measurements) are indicated (* $p \leq 0.01$; ** $p \leq 0.001$).

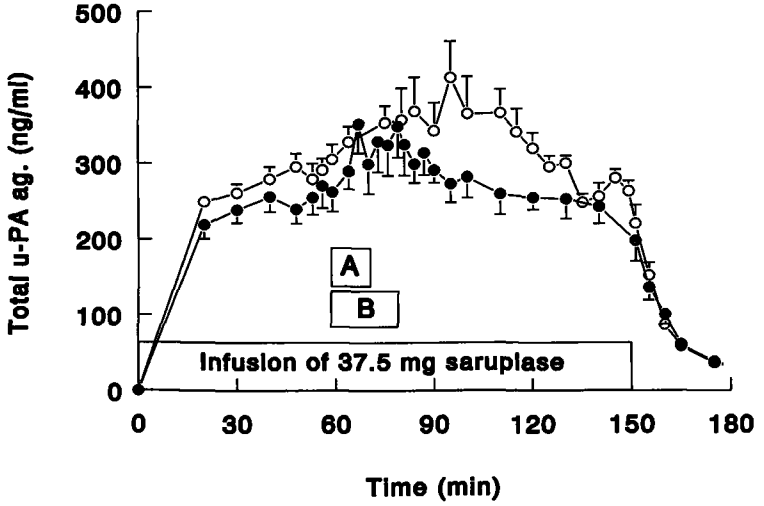

Figure 3. Mean ± SEM (N=10) plasma concentration-time profiles of total urokinase-type plasminogen activator (u-PA) antigen after an infusion of 37.5 mg saruplase over 150 minutes with the consumption of a standard meal (-○-) in 10 minutes (A) after 60 minutes or an exercise test (-●-) over 20 minutes (B). Both experimental interventions were carried out in the same subjects with a 2-week interval.

The results of the coagulation and fibrinolytic parameters are summarized in Table II. An increase in total fibrin and fibrinogen degradation products was observed after administration of saruplase. Reliable statistical analysis of this parameter could not be performed because of occurrence of clotting in a number of the plasma samples. However, the increase appeared to be larger on the day the volunteers had their standardized meal.

Discussion

Exercise increased indocyanine green levels to an extent comparable to the indocyanine green concentrations found in the study of de Boer et al.[31] Therefore it must be assumed that a similar decrease in liver blood flow occurred in both studies. This resulted in an increase in the plasma levels of total u-PA antigen. However, the increase of total u-PA antigen was not as prominent as was observed with recombinant tissue-type plasminogen activator (rt-PA) antigen in the study of de Boer.[31] This variation could have been caused by a difference in metabolism between rt-PA and saruplase. rt-PA is exclusively eliminated by the liver, with a high extraction ratio,[32] whereas saruplase may have a lower extraction ratio than rt-PA and may also be cleared by other organs like the kidney. The data from this study and others[11,12] indicate that the liver is the important organ for the elimination of saruplase. Therefore patients with acute myocardial infarction and impaired liver blood flow caused by cardiogenic shock may have higher plasma u-PA concentrations. The extent of this change in patients cannot be predicted from these data and requires clinical studies.

After the standardized meal an increase in liver blood flow was observed, as reflected in the significant decrease in indocyanine green concentrations. This

Table I. Pharmacokinetic parameters (mean ± SD) of the total urokinase-type plasminogen activator antigen after exercise and after food intake.

Treatment	AUC (ng*min/ml)	CL (ml/min)	$t_{½}\lambda_1$ (min)	$t_{½}\lambda_2$ (min)	V_{ss} (L)
Exercise (N=10)	42235 ± 9852	935 ± 236	5.5 ± 2.9	68.3 ± 37.4	18.4 ± 6.4
Food (N=9)	46129 ± 5013	821 ± 88	4.5 ± 1.9	44.0 ± 14.6	12.0 ± 2.9

Data are mean ± SD.
For subject 4, treatment 2, data could not be fitted. Therefore, no estimations of kinetic parameters are available.
AUC: area under the curve; CL: clearance; $t_{½}\lambda_1$: half-life of the first exponential phase; $t_{½}\lambda_2$: half-life of the second exponential phase; V_{ss}: apparent volume of distribution in steady state.

effect lasted longer than the duration of the saruplase infusion and therefore influenced, to a certain extend, the calculation of the pharmacokinetic parameters (Table I). However, no differences were found when the pharmacokinetic data of the two occasions were compared. Because the elimination of saruplase is influenced by liver blood flow, as shown by the exercise test, a decrease in u-PA concentrations was expected after food intake. However, an increase in u-PA concentrations was observed that could not be explained by pharmacokinetic changes caused by altered liver blood flow. Evidently, food affected the kinetic behaviour of saruplase through a mechanism other than change of liver blood flow. The responsible receptor for the rapid clearance of pro-urokinase and two-chain urokinase is thought to be on parenchymal liver cells,[12] but it remains to be identified. However, recent studies have shown that both molecules bind to low-density lipoprotein receptor-related protein/α_2-macroglobulin receptor (LRP) with high affinities. LRP has been proven responsible for mediation of the internalization and degradation of pro-urokinase in Hep G2 cells.[33] Therefore it is highly likely that this receptor plays an important role in regulating fibrinolytic activity. Food or food components possibly interfere with this process, and it cannot be excluded that food affected another clearance mechanism. It remains to be investigated if the clearance of the thrombolytic rt-PA is similarly affected and if this finding has any clinical implications. In this study no determination of blood levels of u-PA without intervention was performed. As mentioned before, this was done because the amount of blood that was taken from the volunteers had reached a limit set for a participant in a single study (670 ml) and to reduce the exposure of subjects to saruplase. This could be done because the pharmacokinetic properties of saruplase were well known[31] and because interference by endogenous concentrations of u-PA would be minimal at these high concentrations. There was a clear temporal relationship between the

Table II. Maximal changes of the pharmacodynamic parameters after an infusion of 37.5 mg saruplase over 150 minutes and the differences between the areas under the effect curves (AUC; 0-720 min) of the two treatments (food and exercise).

Pharmacodynamic parameter	Treatment	Predose ± SD	minimum value (SD)	95%CI of the difference (δ) between treatment AUC's
α_2-Antiplasmin (%)	Exercise	100	25 ± 17	δ= 5%; -31,+58%
	Food	100	22 ± 18	
Fibrinogen (g/L)	Exercise	1.8 ± 0.4	1.4 ± 0.2	δ= 7%; - 9,+26%
	Food	1.7 ± 0.3	1.2 ± 0.4	
Factor XII-dependent fibrinolytic activity (BAU/ml)	Exercise	34.4 ± 19.3	25.0 ± 10.8	δ= 15%; -14,+53%
	Food	36.7 ± 13.7	17.3 ± 8.0	

interventions and the changes in u-PA antigen concentrations that further supports that these were in fact caused by exercise and food. Moreover, steady state plasma concentrations (C_{ss}) calculated with the formula $C_{ss} = R_0/CL$, in which R_0 is the rate of infusion and CL is the clearance, did not differ from baseline concentrations. With exercise, a calculated C_{ss} of 267 ng/ml was comparable with a baseline value of 263 ng/ml; with food, a C_{ss} value of 305 ng/ml was not different from the 292 ng/ml at baseline. Therefore the interventions (food and exercise) have taken place after reaching steady state.

The change in diameter of the main portal vein and of the portal vein branch were of approximately the same magnitude (13% and 11%) and are in good agreement with data previously reported.[34] The simultaneous change in mean blood flow velocity and diameter in the portal vein branch resulted in an increased blood flow comparable to data previously reported for the main portal vein.[34]

Coagulation was affected by administration of 37.5 mg saruplase. The assumption was made that these parameters would change less on a study day that food was consumed than on a day that an exercise test was performed because it was expected that saruplase concentrations would reach higher levels during exercise than after food intake. However, the standardized meal increased saruplase levels and these levels were on average even higher than during the exercise test. Although the differences were small and not significant, the combination of saruplase and food tended to have a greater influence on coagulation than the combination of saruplase and exercise. The nature of these effects clearly indicated systemic plasminemia, with a reduction of α_2-antiplasmin. However, at this dose of saruplase α_2-antiplasmin is not fully exhausted and the average reduction of fibrinogen remains limited.

In this study saruplase decreased factor XII-dependent fibrinolytic activity in healthy volunteers. This reduction is similar to the effects observed with rt-PA or streptokinase in a study performed by Munkvad et al.,[35] in which both compounds decreased the activity because of an exhaustion of the main proactivator of the fibrinolytic pathway. In another study by Munkvad et al.[36] the incidence of recurrent myocardial infarction was associated with the factor XII-dependent fibrinolytic activity after thrombolytic treatment with rt-PA. Patients in the reinfarction group were characterized by a more pronounced depletion of factor XII-dependent fibrinolytic activity compared with the patients without reinfarction. Although the data from this study do not allow any conclusions regarding the association between the amount of depletion of factor XII-dependent fibrinolytic activity and the development of early recurrent coronary thrombosis, this remains to be investigated with saruplase.

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CHAPTER 4

THE INFLUENCE OF INCREASED LIVER BLOOD FLOW ON THE KINETICS AND DYNAMICS OF RECOMBINANT TISSUE-TYPE PLASMINOGEN ACTIVATOR IN HEALTHY VOLUNTEERS

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Summary

Objective: *To investigate the influence of increased liver blood flow on the pharmacokinetics and pharmacodynamics of recombinant tissue-type plasminogen activator (rt-PA) and to study the changes in endogenous urokinase-type plasminogen activator (u-PA).*

Methods: *This open, randomized, cross-over trial was carried out in a clinical research unit. Eight healthy, non-smoking volunteers received linear infusions of 24 mg rt-PA and 92 mg indocyanine green (ICG) over 160 minutes. Sixty minutes after starting the infusions the subjects consumed a standardized meal to increase liver blood flow on one occasion and abstained from taking food on the other occasion. Plasma concentrations of ICG, t-PA antigen, t-PA activity, total u-PA antigen, plasmin-activatable single chain u-PA (scu-PA), active two chain u-PA (tcu-PA), fibrinogen, total fibrin and fibrinogen/fibrin degradation products (TDP) and α_2-antiplasmin were measured.*

Results: *After the consumption of the meal the area under the curve (AUC) was 35% (95% confidence interval [CI]:+25%,+43%) lower for ICG, 15% (CI:+6%,+24%) lower for t-PA antigen and 11% (CI:+2%,+19%) lower for t-PA activity compared to the AUC after abstaining from food. No changes in fibrinogen, TDP and α_2-antiplasmin concentrations were seen after the intake of food. The infusion of rt-PA caused a five fold increase in the concentration of active tcu-PA and a concomitant decrease in scu-PA concentrations by more than 50%.*

Conclusions: *Increased liver blood flow results in an increase in t-PA clearance, although, as theory predicts, this increase is proportionally smaller. The conversion of the inactive zymogen scu-PA to the active tcu-PA is increased by an infusion of rt-PA but total u-PA antigen concentrations remain unchanged.*

Introduction

Plasmin is an enzyme that dissolves the fibrin of a blood clot. Thrombolytic drugs are activators of the conversion of the inactive proenzyme plasminogen to the proteolytic enzyme plasmin. This property has been studied and clinically been applied in patients with myocardial infarction,[1-4] deep vein thrombosis[5,6] or pulmonary embolism.[7,8] It is generally accepted that thrombolytic therapy is the preferred treatment for myocardial infarction.

Recombinant human tissue-type plasminogen activator (rt-PA) is a thrombolytic agent used for the treatment of acute myocardial infarction. It has a t½ of 5.3 minutes[9] and it is exclusively eliminated from the blood by the liver.[10,11] The variability of plasma concentrations of t-PA in healthy subjects was not high, while a large intersubject variability in steady-state plasma concentrations of t-PA was found in patients after identical rates of infusion.[12-14] Part of this variability can be reduced by the adjustment of the dose for each patient's weight,[15] but an increase or decrease in liver blood flow should also have an influence on the t-PA clearance.

Myocardial infarction could cause a decrease in liver blood flow by impairment of cardiac output. As a result patients could have high t-PA plasma concentrations. A relationship has been found between high t-PA plasma concentrations and early lysis[9] and a higher incidence of bleeding complications in nonsurgical patients.[16] The influence of reduced liver blood flow on the kinetics of t-PA has been investigated by de Boer et al.[17] Liver blood flow was documented indirectly by the clearance of indocyanine green (ICG). A reduction of liver blood flow was induced by performing an exercise test. With an average reduction in flow of 57% an increase in t-PA activity by 119% and t-PA antigen by 91% was observed.

Low t-PA plasma concentrations in patients may result in reduced thrombolytic efficacy. In patients with acute myocardial infarction liver blood flow is correlated with t-PA clearance.[18] In most of these patients liver blood flow is

reduced because of congestive heart failure. Increased liver blood flow in patients can be caused by vasodilating drugs, but can be obtained experimentally by food intake,[19,20] and may result in an increase of the elimination of high clearance drugs. Fat provides the greatest individual stimulus to human intestinal blood flow[21] and a high-protein meal is reported to increase hepatic blood flow.[22,23] Therefore, the objective of this study was to investigate the influence of an increased liver blood flow on the pharmacokinetics and pharmacodynamics of recombinant tissue-type plasminogen activator. Changes in endogenous urokinase-type plasminogen activator induced by the infusion of rt-PA or changed liver blood flow were also studied.

Materials and methods

Subjects

A group of 8 healthy male volunteers (age 23 ± 2 years, length 185 ± 4 cm, weight 82 ± 9 kg) participated in this open randomized cross-over study after informed consent was obtained. None of the subjects had a condition known to be associated with an increased bleeding risk (hypertension, gastro-intestinal erosions or ulcers, the use of antiplatelet drugs or acquired or congenital haemorrhagic diathesis). The volunteers were in good health as judged by medical history, physical examination, twelve-lead electrocardiogram, haematology, blood chemistry, activated partial thromboplastin time, thrombin time, prothrombin time, bleeding time determined with the Ivy method,[24] fibrinogen level, and urinalysis. All test were performed within two weeks before the start of the study. None of the volunteers received concomitant medication or recent medical treatment. The subjects had not participated in a clinical trial during the six months prior to the start of the study, had not donated blood within three months of the start of the study, did not show evidence of a recent trauma (with haematoma) or surgery in the four weeks preceding the study, and were known not to be alcohol or drug dependent. Subjects were studied after abstaining for 48 hours from alcohol and from strenuous physical activities. Caffeine containing beverages, alcohol and smoking were not allowed on the study days. The study was performed according to the principles of the "Declaration of Helsinki" (as amended in Tokyo, Venice, and Hong Kong) and the protocol was approved by the ethics committee of Leiden University Hospital.

Treatments

A total amount of 92 mg indocyanine green (ICG) was given by intravenous infusion (100 mg in 40 ml aqueous solvent) over a time period of 160 minutes

(0.575 mg/min) and simultaneously a total amount of 24 mg recombinant tissue-type plasminogen activator (rt-PA) was administered intravenously (0.15 mg/min).

ICG is a tricarbocyanine dye which has been extensively used to estimate liver blood flow because of its physical properties.[25-28] It is rapidly distributed in the circulating plasma and does not reach extravascular compartments. It is exclusively removed by the liver without biotransformation[29] and does not undergo an enterohepatic circulation. The hepatic extraction ratio in man is high[30] (usually 0.7 or greater) and therefore its clearance is related to liver blood flow.

The syringes with the required amounts of rt-PA (alteplase, Boehringer Ingelheim, Germany) and ICG (Cardiogreen^{R}; Hynson, Westcott & Dunning, Balimore, MD, USA) were prepared by Leiden University Hospital Pharmacy. All the medication was administered using syringe infusion pumps (Harvard Apparatus syringe infusion pump, model 22, South Natick, Mass., USA). An interval of one week separated the two study periods.

The subjects arrived at the study centre after an overnight fast, beginning latest at midnight of the preceding day. The volunteers were not allowed to be in a supine position but were asked to remain sitting during the entire experiment. Two intravenous cannulas (Venflon 18G) for administration of rt-PA and ICG were inserted in forearm veins of the same arm. A third cannula was inserted in the contralateral arm for collection of the blood samples and this cannula was kept patent by a continuous infusion of saline. Blood chemistry, haematology and urine samples were taken and before administration of the medication the volunteers rested for 90 minutes. Sixty minutes after starting the infusions the subjects either consumed a standard meal in 10 minutes, the time being noted when the subjects finished their meal, or remained fasting during the rest of the experiment. The standard lipid/protein rich meal consisted of 600 kcal (25 energy % protein, 51 energy % lipid and 24 energy % carbohydrate). The infusions of rt-PA and ICG were stopped after 160 minutes and after 300 minutes the last blood samples were collected, the volunteers were allowed food and drinks and the cannulas were removed. Twelve hours after the start of the infusions the subjects returned home. At the end of the last occasion safety blood and urine samples were taken.

Blood sampling

Blood was collected after the saline infusion was interrupted and after discarding the contents of the cannula. A total amount of 440 ml was collected over a time period of two weeks. Blood samples of 3 ml were collected for the determinations of ICG in lithium heparin tubes (Sarstedt, Nümbrecht, Germany) and for the t-PA and u-PA assays 4.5 ml was collected in ice-cold Stabilyte vacutainer tubes (0.5 ml acidic citrate; Biopool, Umeå, Sweden) shortly before

and 45, 50, 56, 59, 65, 70, 75, 80, 85, 90, 95, 102, 109, 116, 123, 130, 137, 144, 151 and 159 minutes after the start of the infusions. Determinations of fibrinogen, total fibrin and fibrinogen/fibrin degradation products (TDP), and α_2-antiplasmin were performed after collecting 3 ml of blood in Monovette^R citrate tubes (Sarstedt, Nümbrecht, Germany) containing prostaglandin E_2, theophylline and Glu-Gly-Arg-Chlorometyl-ketone) just before administration and after 59, 102, 180, 240 and 300 minutes. After collecting the blood all samples were immediately put on ice and subsequently centrifugated at 5000 g at 4°C for 6 minutes. After centrifugation the platelet poor plasma was separated and stored for the ICG assay at -40°C and for the other assays snap frozen and stored at -70°C until analysis.

Assays

Plasma samples of indocyanine green were determined by high performance liquid chromatography (HPLC) according to Rappaport & Thiessen[31] in a slightly modified form using diazepam as internal standard. The assay had a detection limit of 0.1 μg/ml. Plasma concentrations of t-PA antigen were determined by an enzyme-linked immunosorbent assay technique.[32] For the activity assay plasma samples were acidified to neutralize inhibitors and the plasmin generation was determined by a spectrometric method.[33] Endogenous levels of total u-PA antigen were determined with an ELISA as described by Binnema et al.[34] The assay measures the inactive proenzyme scu-PA, the active tcu-PA and u-PA complexed with inhibitors with equal efficiency. The concentration of plasmin-activatable scu-PA and of tcu-PA were measured by BIA.[35] Calibration was performed by scu-PA purified from human fibroblasts. The lower limit of sensitivity of these assays is 0.1 ng/ml for 50 μL plasma samples. The α_2-antiplasmin was determined by an immediate plasmin inhibition test using H-D-Val-Leu-Lys-pNA as plasmin substrate according to an improved automated method of Friberger.[36] The detection limit of the assay was 12.5% of the normal plasma concentration. Fibrinogen was measured according to Clauss (detection limit of 0.5 g/l)[37] and fibrinogen, total fibrin and fibrinogen/fibrin degradation products were measured with an enzyme-linked immunosorbent assay method according to Koopman (detection limit of 5 ng/ml).[38]

Data processing

For each volunteer an estimation of the changes in liver blood flow were assessed by measuring the plasma concentrations of ICG. The concentrations of ICG, t-PA antigen and t-PA activity were analyzed by calculating the areas under the curve (AUC) by linear trapezoidal rule covering the time from the start of intervention to the end of infusion. The AUC's were analyzed using

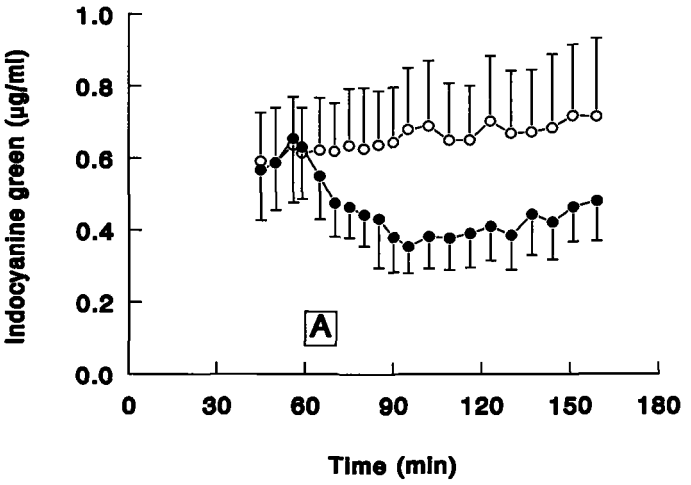

Figure 1. Average ± SD indocyanine plasma concentration-time curves with (-●-) and without (-○-) the consumption of a standard meal (A) in ten minutes, sixty minutes after the start of the infusion.

paired t-tests after log-transformation. The back-transformed log-differences provided estimations of the percentage of decrease in AUC with 95% confidence intervals (CI) caused by the consumption of a standard meal.

The effect of exogenously infused rt-PA and the effect of food intake on endogenous total u-PA antigen, plasmin-activatable single chain u-PA and active tcu-PA concentrations were measured. The t-PA infusion effect was analyzed by comparing the average of the two treatments at the time just before the start of the infusions (t= 0 minutes) to the average of the two treatments at the time just before intake of the meal (t= 59 minutes). The food effect was analyzed by comparing the two treatments at t=130 minutes, and additionally by comparing the change from 59 to 130 minutes for both treatments separately. All data were analyzed using paired t-tests and results were reported as percentages change along with their CI.

Fibrinogen, TDP and α_2-antiplasmin were analyzed using Repeated Measures ANOVA. In order to assess the effect of the t-PA infusion, paired t-tests with the pre-value were performed in the presence of a significant time-effect. In the absence of a significant treatment or treatment by time effect, average time responses over the two treatments were used for the t-tests. Data were log-transformed prior to analysis. Percentage change from pre-value were calculated using the back-transformed log-differences. Paired t-tests and Repeated Measures ANOVA were performed using SPSS/PC+ V4.0.1 (SPSS, Inc., Chicago, IL, USA). AUEC's were calculated using BMDP/Dynamic Release 7.0 (BMPD Statistical Software, Inc., Los Angeles, CA, USA).

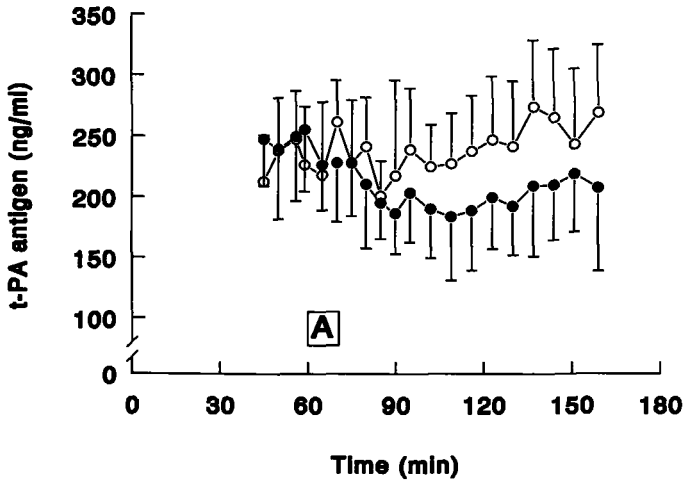

Figure 2. Average ± SD t-PA antigen plasma concentration-time curves with (-●-) and without (-○-) the consumption of a standard meal (A) in ten minutes, sixty minutes after the start of the infusion. A similar profile for t-PA activity levels was found for both treatments.

Results

During the study no relevant adverse events were observed. The mean ICG plasma concentration-time profiles for both treatments are presented in Fig. 1. After consumption of the standard meal the AUC was 35% (CI:+25%,+43%) lower compared to the AUC after abstaining from food. Simultaneously, decreases in AUC were observed after the intake of the standard meal for t-PA antigen of 15% (CI:+6%,+24%) and for t-PA activity of 11% (CI:+2%,+19%). The average t-PA antigen concentration curve is presented in Fig. 2.

The pharmacodynamic parameter α_2-antiplasmin demonstrated a significant decrease in time, but no differences between the two treatments could be detected. A maximum decrease of 23% ($p<0.05$) compared to pre-dose value was found 3 hours after the start of the infusion (Fig. 3). The last measured value at t=300 minutes was not significantly different from the average pre-dose value. For fibrinogen no differences could be detected between the two treatments and during the experiment fibrinogen concentrations did not differ from pre-dose. For TDP the concentration-time profile was similar for both treatments but all measured values were significantly increased compared to the pre-medication value with a maximum at t=180 minutes (Fig. 3).

The rt-PA infusion did not affect endogenous u-PA antigen levels. However, in the first 59 minutes of infusion the concentration of active tcu-PA increased

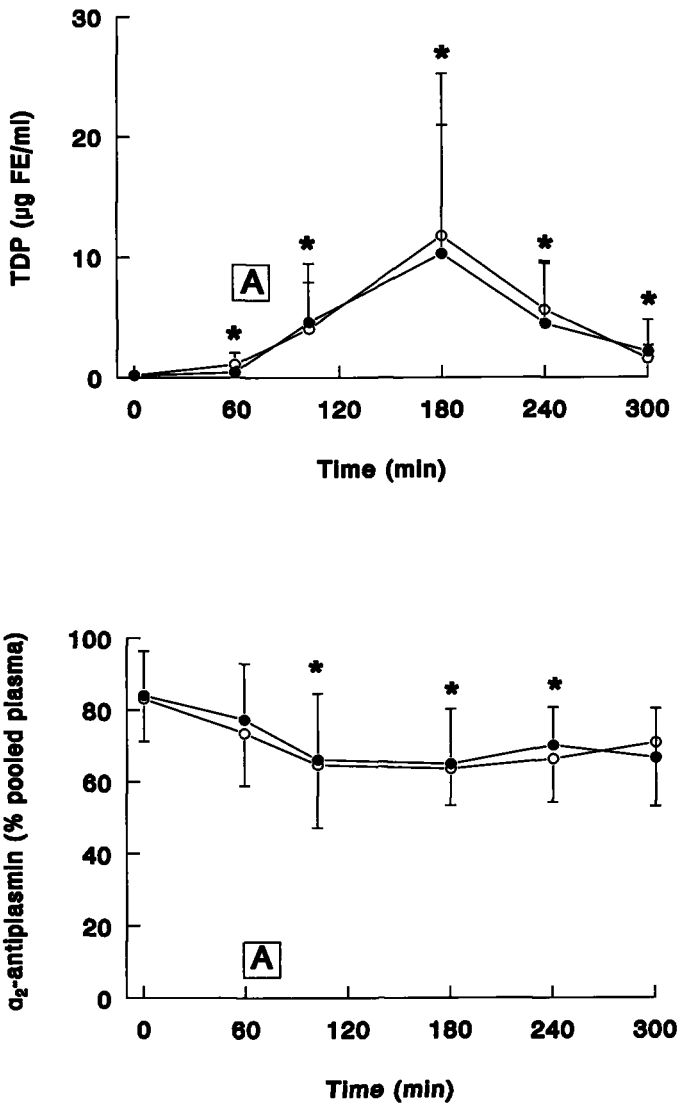

Figure 3. Average ± SD total fibrin and fibrinogen/fibrin degradation products (TDP; upper panel) and α_2-antiplasmin (lower panel) concentration-time curves with (-●-) and without (-○-) the consumption of a standard meal (A). Significant changes to pretreatment values are indicated (* $p \leq 0.05$).

more than five fold and the scu-PA concentration decreased by 60% over the same time period (Fig. 4). A comparison of the two treatments 70 minutes after the intake of the standard meal demonstrated that food did not effect total u-PA antigen, plasmin-activatable single chain u-PA and active tcu-PA levels. However, after food intake a significant reduction of both total u-PA antigen levels and concentrations of plasmin-activatable single chain u-PA were detected when concentrations just before and 70 minutes after the meal were compared.

Discusion

Changes in liver blood flow were reflected rapidly in the t-PA plasma concentrations. As theory predicts[39] an increase in liver blood flow does not affect plasma concentrations as much as a decrease. A 35% reduction in the AUC of ICG after the consumption of a meal is followed by a smaller reduction in AUC of 15% for t-PA antigen and 11% for t-PA activity. In an experiment where liver blood flow was reduced approximately by half during an exercise test, it produced a rapid two- to threefold increase in plasma concentration of exogenously infused rt-PA, in parallel with indocyanine green concentrations.[17] The receptor-mediated mechanism that clears t-PA by the liver appears not to be easily saturated.

This study demonstrates that increased liver blood flow may be the cause for low thrombolytic drug levels. Increased liver blood flow may be caused by certain vasoactive drugs, but also by improvement in cardiac function which may occur after succesful thrombolysis. There has been a paucity of drug interaction studies with t-PA. This is surprising in view of the relatively low safety of this compound with regard to intracranial bleeding en the large number of vasoactive drugs that are generally administered to patients in a coronary care unit. The influence of concomitant vasoactive medication on the pharmacokinetics of t-PA should be studied, as well as the ability of these drug to generate haemodynamic changes in the liver. Our study suggests that the effect on t-PA levels of an increase in liver blood flow over the normal value is likely to be limited. However, this may not be the case when a reduced liver blood flow is increased to the normal value, for example, when an initially depressed cardiac output is increased by inotropic support. In such a case rapid and considerable reductions in t-PA concentrations may occur.

The exogenously infused rt-PA administered in this study had no effect on fibrinogen levels. When higher dosages are applied a decrease in fibrinogen levels are to be expected.[40] The influence of t-PA on the fibrinolytic system was reflected in a significant increase in total degradation products and decrease in α_2-antiplasmin. However, the effect was short and 140 minutes after the end of infusion α_2-antiplasmin levels were back to pre-dosing levels. Although marked decreases in t-PA antigen and activity concentrations were observed after the intake of food, this change was apparently too small or to short lasting to have

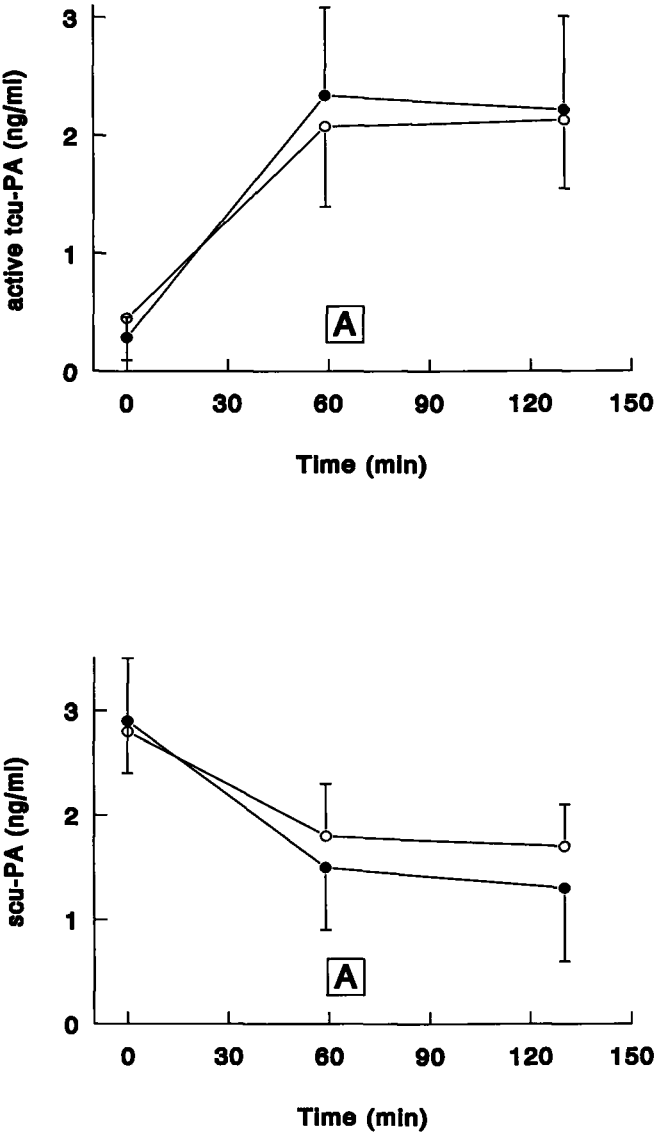

Figure 4. Average ± SD active tcu-PA (upper panel) and scu-PA (lower panel) concentration-time curves with (-●-) and without (-○-) the consumption of a standard meal (A).

an impact on fibrinogen, TDP and α_2-antiplasmin concentrations.

Plasma concentrations of tcu-PA in volunteers increased after receiving an rt-PA infusion. More plasmin is formed by an increase in the conversion of plasminogen into plasmin, while the inactivation rate of plasmin by α_2-antiplasmin remains the same. The α_2-antiplasmin concentration could even decrease because of the extensive reaction with plasmin. Therefore, steady state plasmin concentrations will increase. More plasmin is available to activate scu-PA to tcu-PA resulting in decreased scu-PA concentrations and increased tcu-PA concentrations. An increase in tcu-PA concentrations could also be caused by the inability of tcu-PA to react with plasminogen activator inhibitor 1 (PAI-1). This inability is caused by very low PAI-1 concentrations because most of the PAI-1 has reacted with rt-PA that is present in large quantities.

The clear increase in liver blood flow induced by the lipid/protein rich meal did not produce a distinct effect on the endogenous u-PA concentrations. No differences between the treatments could be found 70 minutes after the consumption of the meal. A small decrease in u-PA antigen and scu-PA concentration was found comparing values just before and 70 minutes after the intake of food, but this comparison does not validate the conclusion that the difference is exclusively produced by the intake of food. The effect of food on u-PA levels is certainly not comparable to the pronounced effect of food on t-PA concentrations, indicating different regulatory mechanisms for u-PA and t-PA. The discrepancy in effects of changed liver blood flow between u-PA and t-PA has been described in previous studies. The influence of food and exercise were investigated after an infusion of unglycosylated recombinant single chain urokinase-type plasminogen activator in healthy volunteers.[41] Plasma concentrations were increased by a reduction of liver blood flow induced by an exercise test, but food intake produced an unexpected increase in u-PA concentrations as well, despite increases in liver blood flow. However, this effect might have been caused by the administration of the unglycosylated scu-PA instead of the native glycosylated form. Although in studies that observe the exercise-related changes in plasma concentrations of plasminogen activators both t-PA and u-PA plasma levels increase during exercise, the increases in u-PA antigen are also smaller than those of t-PA antigen and do not correlate.[42] These observations again suggest that independent mechanisms regulate the plasma levels of u-PA and t-PA.

Acknowledgements

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Section III

PHARMACOLOGICAL STUDIES IN PATIENTS WITH ACUTE MYOCARDIAL INFARCTION

CHAPTER 5

EFFECT OF CHANGES IN LIVER BLOOD FLOW ON THE PHARMACOKINETICS OF SARUPLASE IN PATIENTS WITH ACUTE MYOCARDIAL INFARCTION

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Submitted for publication

Summary

Background: *The recombinant unglycosylated single chain urokinase-type plasminogen activator saruplase is cleared for a large part by the liver. A large interindividual variation in saruplase concentration is found in acute myocardial infarct (AMI) patients. The variable cardiac performance after an infarct may induce differences in liver blood flow that could explain the concentration diversity. This study was performed to investigate the relation between hepatic blood flow and the pharmacokinetic and pharmacodynamic properties of saruplase.*

Methods and Results: *Thirteen AMI patients were enroled in this open label study. Patients received a bolus injection of 20 mg saruplase followed by a one-hour infusion of 60 mg saruplase. Concurrently 36 mg intravenous indocyanine green (ICG) was given over 1 hour to measure hepatic blood flow. Blood samples were taken at regular time intervals to measure plasma levels of*

urokinase-type plasminogen activator (u-PA) antigen and activity, the two-chain form (tcu-PA) activity, indocyanine green, fibrinogen, fibrin and fibrin degradation products, α_2-antiplasmin and thrombin antithrombin III complex. A correlation was seen between the clearance of ICG and both those of u-PA antigen ($r = 0.62$; $p<0.05$) and u-PA activity ($r = 0.57$; $p<0.05$). The area under the curve of tcu-PA activity and the area under the effect curve of fibrinogen and α_2-antiplasmin were negatively correlated ($r = -0.84$; $p<0.01$ and $r = -0.65$; $p<0.05$).

Conclusions: *Liver blood flow is an important determinant of the clearance of u-PA antigen and activity and reduction of flow in patients with heart failure will lead to an increase in plasma concentrations. The plasma concentrations of tcu-PA activity are associated with systemic fibrinogenolysis. These results may be used to optimize saruplase treatment in patients with impaired cardiac function or after co-medication with drugs that affect liver blood flow.*

Introduction

The recombinant unglycosylated single chain urokinase-type plasminogen activator (scu-PA) saruplase is under investigation as a thrombolytic in patients with acute myocardial infarction (AMI).[1-5] Its efficacy, in terms of patency rate and mortality rate, is comparable to other thrombolytics.[1] Saruplase directly converts plasminogen to plasmin[6] and is converted by plasmin to a two chain form (tcu-PA; urokinase[7]) by hydrolysis of the Lys 158-Ile159 peptide bound.[8] Saruplase is cleared rapidly from the plasma[9,10] with a half-life of 5-10 minutes and there are cellular receptors in the liver that bind to scu-PA.[11] Although the exact elimination routes of saruplase are not known, it is likely that extraction in a single pass through the liver is so high that the blood flow to the liver becomes rate determining for its clearance, as is the case with tissue plasminogen activator (t-PA).[12] The liver exclusively eliminates t-PA from the blood[13-15] and changes in liver blood flow affect its concentration. Lidocaine is a well known drug for which hepatic blood flow is rate determining for its clearance. Patients with reduced cardiac function have significantly higher steady state lidocaine levels caused by a reduced lidocaine clearance which is due to the reduction in liver blood flow.[16-18]

Large interindividual differences of saruplase concentrations in patients with a myocardial infarction have been reported[19] and may have important clinical implications. Concentrations of saruplase that are too low could possibly result in insufficient thrombolysis. Concentrations of the thrombolytic drug that are too high could lead to an increased bleeding risk. Therefore, we performed this study to investigate the correlation between the pharmacokinetics and pharmacodynamics of saruplase and hepatic blood flow in patients with AMI. As a secondary objective we investigated if patients with a marked reduction in liver blood flow could be detected by clinical signs.

Methods

Patients

Thirteen patients with acute myocardial infarction participated in this open label study after informed consent was obtained. All patients were treated at a single centre. Patients with pain compatible with an evolving myocardial infarction for at least 30 minutes despite the use of sublingual nitrates were included. The electrocardiographic changes had to be diagnostic for a myocardial infarction. For anterior infarction the sum of ST-segment elevation had to be $\geq$ 0.6 mV in V1 - V6 and for inferior infarction the sum of ST-segment elevation was $\geq$ 0.6 mV in II, III, AVF, V5 and V6 or the sum of ST-segment elevation in II, III, AVF, V5 and V6 plus ST-segment depression in V1 - V4 $\geq$ 1.2 mV.

Thrombolytic therapy had to be started within 6 hours after the onset of pain and the standard exclusion criteria for thrombolytic therapy were applied. The study protocol was approved by the Ethical Committee of the Academic Medical Center, Amsterdam.

Treatments and interventions

Thrombolytic therapy was started with a bolus injection of 20 mg of saruplase (Grünenthal GmbH, Aachen, Germany) and followed by an infusion of 60 mg in 1 hour. To estimate hepatic blood flow 36 mg indocyanine green (ICG) was administered over 1 hour simultaneously with the infusion of saruplase.

ICG (Cardiogreen^{R}; Hynson, Westcott & Dunning, Baltimore, MD, USA) is a tricarbocyanine dye which has been extensively used to estimate liver blood flow because of its physical properties.[20-23] It is rapidly distributed in the circulating

Table I. Clinical characteristics of the patients

- Sex: Male/Female	9/4
- Age (years)	59 $\pm$ 7
- Weight (kg)	75 $\pm$ 16
- Infarct location: Anterior/inferior	4/9
- First/recurrent	13/0
- Killip classification at admission: I/II/III/IV	11/2/0/0
- Time interval between onset of symptoms and treatment: 0-2/2-4/4-6 hours	3/8/2

plasma and not in the extravascular compartments. It is exclusively removed by the liver without biotransformation[24] and does not undergo an enterohepatic circulation. The hepatic extraction ratio in man is high[25] ($\geq$ 0.7) and therefore its clearance is similar to liver blood flow.

A bolus of 5000 IU heparin was given before starting fibrinolytic medication and an infusion of 1000 IU/h heparin was started as soon as possible and continued for 48 hours with adjustment of the activated partial thromboplastin time by 1.5 - 2 times the normal value. Acetylsalicylic acid was given orally as a 300 mg loading dose before the start of the thrombolytic medication and continued orally. Clinical management of the patients was according to routine coronary care in our institution.

Experimental protocol

After arrival in the hospital and determination of eligibility two intravenous cannulas were inserted into a forearm vein of the same arm for separate administration of saruplase and ICG and a third cannula was inserted for blood collection in the contralateral arm. The cannula for blood collection was kept patent by a continuous infusion of saline without the addition of heparin. For the pharmacokinetic assays 4.5 ml of blood was collected in 5 ml tubes (Monovette^{R}; Sarstedt, Nümbrecht, Germany) containing sodium citrate (0.5 ml of 0.129 mol/L) shortly before and 2, 6, 16, 31, 41, 51, 56, 63, 65, 67, 71, 76, 81, 91, 120, 180 and 240 minutes after start of the bolus injection. The content of the Monovette^{R} was transferred into an 8 ml reagent tube containing 250 μl aprotinin (6000 KIU/ml) and benzamidine (1 mol/L). For the ICG assay 3 ml of blood was collected in tubes containing lithium heparin (4.5 ml Monovette^{R}) at the same time points as for the pharmacokinetic parameters accept at 180 and 240 minutes. For the haemostatic assays 4.5 ml of blood was collected in tubes (5 ml Monovette^{R}) containing sodium citrate (0.5 ml of 0.129 mol/L) just before administration of the trial medication and after 31, 56, 120, and 240 minutes. The content of the Monovette^{R} was then transferred into an 8 ml reagent tube containing 250 μl GGACK (Glu-Gly-Arg-chloromethylketone; 200 μmol/L) as a stabilizer. Blood was taken after the saline infusion was interrupted and after discarding the contents of the cannula. The sampled venous blood was centrifuged at 3000 g at 4°C for 10 minutes, snap frozen and stored at -20°C. The Killip classification[26] of the patients was determined at arrival in the hospital and 61 and 240 minutes after the start of the infusion.

Assays

Total u-PA antigen (saruplase and degradation products) was determined by means of an ELISA technique.[27] The limit of quantitation of the assay was

2 ng/ml. High molecular weight u-PA (HUK) antigen was determined by means of an ELISA technique. This assay only determines u-PA molecules which contain the kringle domain as well as the enzymatic domain of saruplase. The assay did not differentiate between single- and two-chain u-PA. An immuno-activity assay (IAA) with Glp-Gly-Arg-pNA substrate (S-2444, Kabi Diagnostica) was used to measure the two chain form (tcu-PA) activity before and after activation with plasmin.[27] The assay without plasmin activation determines the in vivo tcu-PA activity protein equivalents which will be referred to as tcu-PA activity. The assay with plasmin activation includes the single-chain protein saruplase which is converible to the two-chain form urokinase; this activity will be referred to as total u-PA activity. The detection limit of two-chain urokinase plasminogen activator activity assay (IAA) was 3.7 ng/ml. The plasma levels of indocyanine green were determined by a high performance liquid chromatographic (HPLC) method according to Rappaport and Thiessen[28] in a slightly modified form using diazepam as internal standard. The detection limit of the method was 0.1 μg/ml. Fibrinogen was determined in the plasma samples essentially according to Clauss.[29] The mean of at least two valid measurements was evaluated. Values are given in mg/dl citrated plasma. The lower detection limit of this assay was 47 mg/dl citrated plasma. Total fibrin and fibrinogen degradation products (TDP) were determined by an ELISA technique (Organon Teknika, Eppelheim) that could equally detect both types of degradation products. The mean of at least two valid measurements was used. Values are given in μg of fibrinogen equivalents (FE) per ml citrated plasma. The α_2-antiplasmin-dependent plasmin-inhibiting capacity of plasma samples was determined by means of a Behrichrom α_2-antiplasmin test kit (Behringwerke AG, Marburg). Values of functional α_2-AP are given in per cent of the normal value. The mean of at least two valid measurements was evaluated. Antigen of the thrombin antithrombin III complex (TAT) was determined in the plasma samples by means of an ELISA using the Enzygnost-TAT micro kit (Behringwerke AG, Marburg). The mean of at least two valid determinations was evaluated. Values are given in μg/L as calibrated by the standards of the test kit.

Data analysis

Where appropriate pre-treatment values were subtracted from subsequent time points to correct for endogenous levels. Pharmacokinetic parameters were calculated using a standard two-compartment pharmacokinetic model for total u-PA antigen, HUK antigen and total u-PA activity, while tcu-PA activity was evaluated with a model independent analysis. The area under the curve (AUC), clearance (CL), half-life of the first exponential phase ($t_{½,\lambda1}$), half-life of the second exponential phase ($t_{½,\lambda2}$), volume of distribution at steady state (Vd_{SS}) and mean residence time (MRT) were computed using the fitted models (Siphar software package; Simed, Créteil, France). The AUC with its corresponding

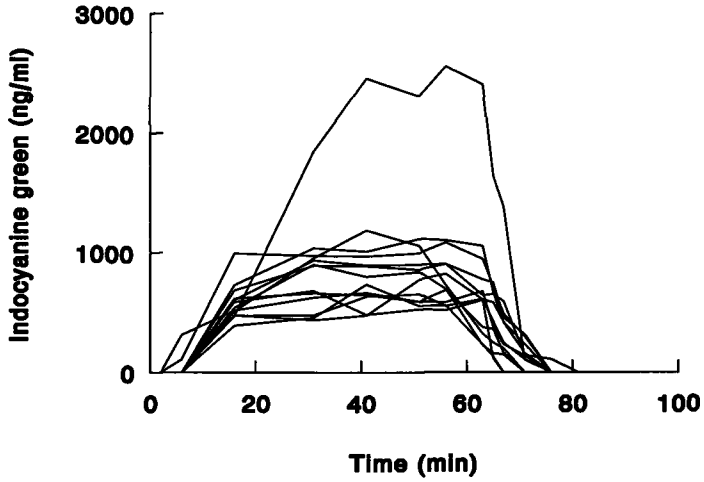

Figure 1. Individual plasma curves of indocyanine green (36 mg ICG in one hour). Note the large interindividual differences; the patient with the hypotensive crisis shows a different profile.

clearance (D/AUC) were also determined by means of the trapezoidal rule without extrapolation to infinity (AUC_{0-t}). The conversion of saruplase to its two-chain form was calculated by the ratio of the AUC's of saruplase and the two-chain form. Pearson's correlation coefficients were calculated between total u-PA antigen clearance and indocyanine green clearance and between total u-PA activity clearance and indocyanine green clearance. Model independent clearances were used for these comparisons. Correlation coefficients were also calculated between the area under the effect curves (AUEC) of haemostatic parameters and AUC's of tcu-PA. Results are given as means ± SD. Values were considered significantly different when $P < 0.05$.

Results

The clinical characteristics of the 13 analyzable patients are summarized in Table 1.

Pharmacokinetics of indocyanine green and saruplase

Peak plasma ICG levels varied from 652 ng/ml to 2556 ng/ml (Fig. 1). The clearance differed accordingly from 1350 ml/min to 343 ml/min. Plasma concentrations for total u-PA antigen are displayed in Fig 2a. Interindividual

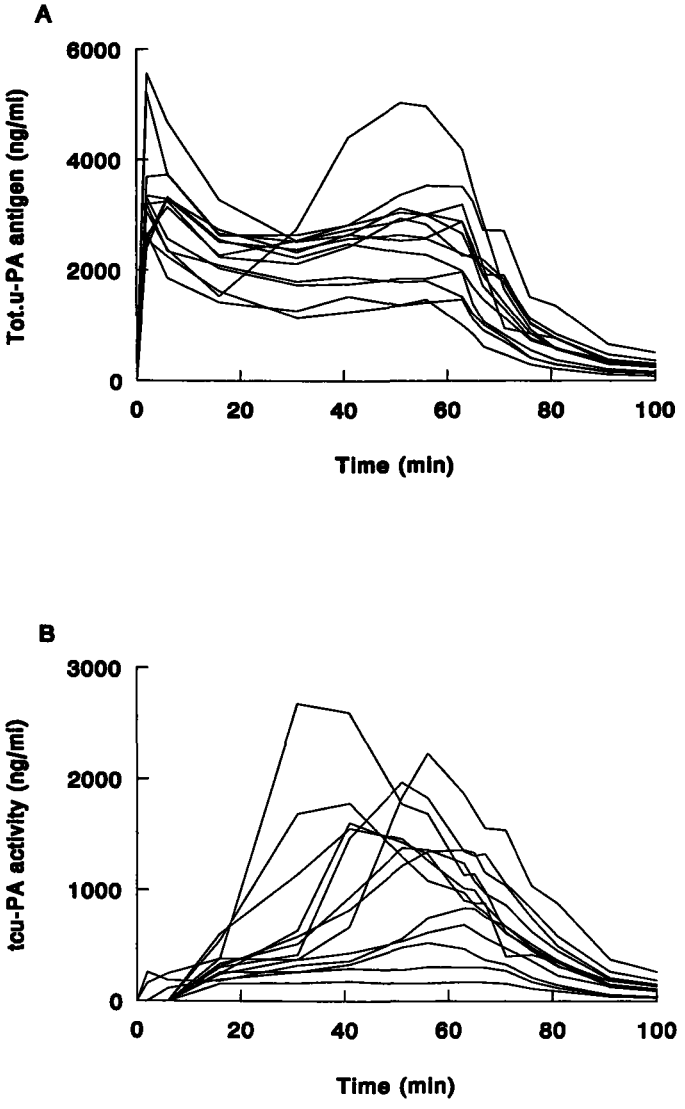

Figure 2. Individual plasma curves of total u-PA antigen (A) and tcu-PA activity (B) after a bolus injection of 20 mg saruplase followed by an infusion of 60 mg; the patient with a hypotensive crisis shows a different profile.

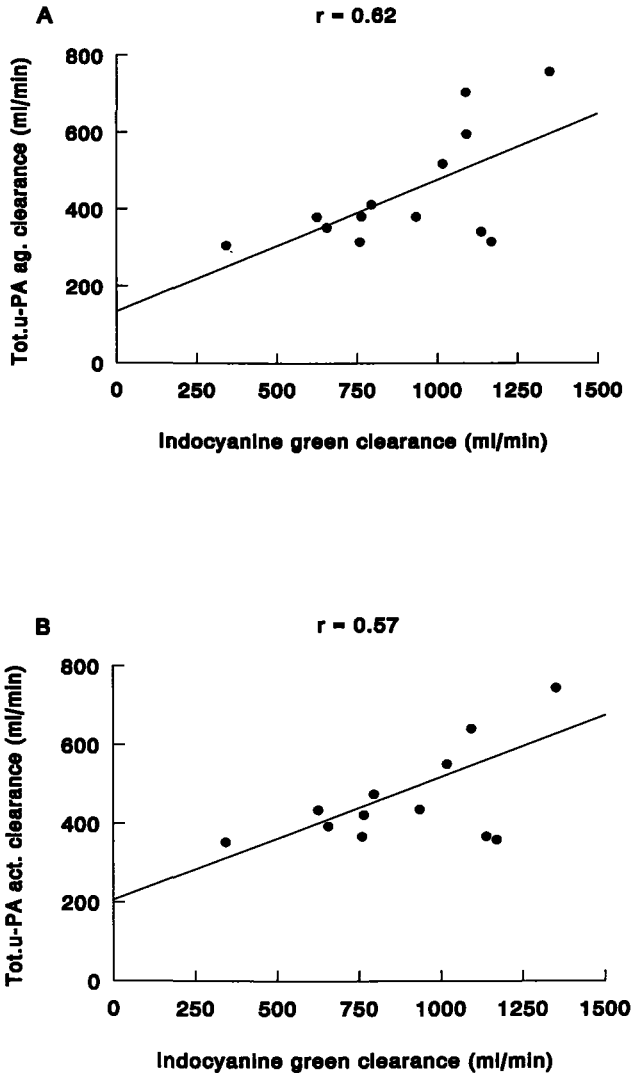

Figure 3. Relationship between ICG clearance (liver blood flow) and the clearance of total u-PA antigen (A) and total u-PA activity (B). The clearance is calculated by dividing the administered dose by the area under the concentration curve.

differences in clearance were 307 ml/min to 758 ml/min for total u-PA antigen, 284 ml/min to 647 ml/min for HUK antigen, and 353 ml/min to 803 ml/min for total u-PA activity. Peak values of tcu-PA activity ranged from 178 to 2679 ng/ml (Fig. 2b). The pharmacokinetic parameters derived from the individual curves are displayed in Table 2. The percentage conversion of saruplase to urokinase, estimated under the assumption of identical kinetic behaviour, ranged from 10.3 to 42.9 %. There was a significant correlation (Fig. 3) between ICG clearance, as a measure for hepatic blood flow, and clearances of both total u-PA antigen ($r = 0.62$; $p<0.05$) and total u-PA activity ($r = 0.57$; $p<0.05$). One patient developed hypotension during infusion and the resulting impaired liver blood flow was reflected in the extremely high ICG concentrations and a 2-3 fold elevation of saruplase levels during the period of hypotension (Fig. 4).

Fibrinolytic parameters

The fibrinogen concentration before thrombolytic treatment was 344 ± 140 mg/dl with total fibrin and fibrinogen degradation product concentrations of 2.0 ± 1.8 μg FE/ml. The average lowest fibrinogen level of 69 ± 37 mg/dl was at the last measurement 4 hours after drug administration. A negative correlation was observed (Fig 5a) between the area under the effect curve (AUEC) of fibrinogen and the tcu-PA activity AUC ($r = -0.84$; $p<0.01$). Peak fibrin and

Table II. The pharmacokinetic parameters (mean ± SD). For subject 7 no model dependent analysis could be performed.

	ICG†	u-PA antigen	u-PA activity	tcu-PA activity*
AUC (0-t) (ng*h/ml)*	45 ± 20	3282 ± 892	2936 ± 744	985 ± 504
CL (0-t) (ml/min)*	903 ± 271	444 ± 152	489 ± 153	
AUC (ng*h/ml)	48 ± 21	3192 ± 856	2883 ± 729	
CL (ml/min)	853 ± 259	454 ± 152	498 ± 156	
$t_{½,λ1}$ (min)		9 ± 2	8 ± 1	
$t_{½,λ2}$ (min)	6 ± 3	70 ± 14	112 ± 50	9 ± 2
Vdss (L)	6 ± 2	9 ± 3	9 ± 3	
MRT (min)	38 ± 5	23 ± 4	19 ± 3	23 ± 5

* Model independent values

† The unit for both model dependent and model independent AUC's for indocyanine green is μg*min/ml

fibrinogen degradation product concentrations were observed 56 minutes after drug administration but also remained elevated after 4 hours. The patient with hypotension and the high saruplase concentrations had extremely high total degradation product concentrations up to 6690 μg FE/ml after 56 minutes. The functional α_2-antiplasmin decreased to 15 $\pm$ 7% of the normal value after 56 minutes and had only returned to 25 $\pm$ 7% 4 hours after saruplase administration. A negative correlation was seen (Fig. 5b) between the AUEC of α_2-antiplasmin and the tcu-PA activity AUC ($r = -0.65$; $p<0.05$). Thrombin antithrombin III (TAT) complexes increased from a pre medication concentration of 17.0 $\pm$ 12.6 μg/L to a concentration of 76.4 $\pm$ 154.4 μg/L after 56 minutes. No correlation between tcu-PA AUC and the AUEC of total fibrin and fibrinogen degradation products and thrombin-antithrombin III complex can be observed.

Clinical observations

At admission in the hospital 11 patients in this study were in Killip I and 9 of these patients remained in this category during the first 4 hours after starting the thrombolytic drug administration. Severe depression of liver blood flow (indocyanine green concentrations) appeared to coincide with elevated Killip class. Killip class II at admission and a classification of IV at 61 minutes after start of the medication was coincidental to a decreased indocyanine green clearance, with a decreased total u-PA antigen and activity clearance.

Discussion

This study has demonstrated that the clearance of saruplase is affected by liver blood flow. This implies that saruplase is very efficiently extracted in single pass through the liver and therefore the liver blood flow becomes rate determining for its clearance. As the half life of saruplase is relatively short, changes in liver perfusion during the infusion are quickly translated into changes in steady state concentrations as was demonstrated by the patient who became hypotensive during infusion. The range of liver blood flow observed in our patients was comparable with the work performed by Zito et al.,[17] although in their study, which was performed in the pre-thrombolytic period, a large group with severe congestive heart failure was included. Liver blood flow is an important determinant of the plasma concentrations of saruplase but not the only one as only approximately 35% of the variance in plasma concentrations is explained by the liver blood flow. Other sources of variability are likely to be numerous and may include plasma volume, body weight, interactions with physiological inhibitors and variability in the assay. In view of the limited number of patients in the study it is not possible to draw conclusions about factors like

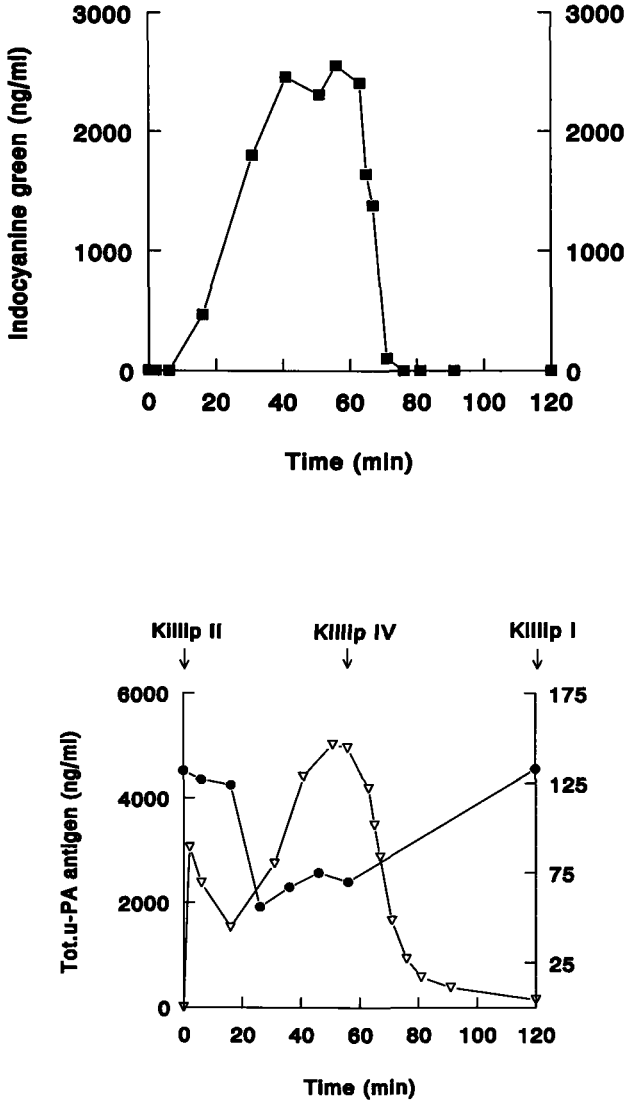

Figure 4. Data of a single patient who became hypotensive (Killip IV) during the infusion of saruplase (mean arterial pressure: -●-). Total u-PA antigen plasma levels rose accordingly (-▽-) and this rise was associated with increased ICG levels (-■-).

hypertension, diabetes mellitus, smoking, sex or age. In AMI patients who received rt-PA treatment these factors had no predictive value for the occurrence of bleeding complications.[30]

The clinical relevance of these findings is obviously strongly dependent upon the importance of plasma concentrations of saruplase for its efficacy and safety. No data about this from sufficiently large studies is available for saruplase. Major bleeding complications do occur more in patients with the highest plasma rt-PA antigen levels[31] but this does not necessarily imply that this also holds for saruplase. However, assuming that some relation between plasma concentrations, efficacy and safety exists, a large variability in plasma concentrations will lead to increased proportions of patients with concentrations that are too low or too high. If a maximum effect exists, the consequence of this in a clinical trial will be reduced efficacy as well as an increase in side effects. There is some evidence that the concentration-effect relationship of saruplase has a maximum. The thrombolytic efficacy of saruplase is dose dependent.[5] But since its thrombolytic action is dependent on the presence of activatable plasminogen, a limitation of its activity may occur. Therefore higher saruplase doses will probably result in an increase in systemic fibrinogenolysis and depletion of α_2-antiplasmin rather than in increased thrombolytic efficacy.[32] High concentrations of saruplase lead to a clear increase in systemic fibrinogenolysis, caused by increased systemic plasminogen activation and formation of the two-chain form.[19] The correlation between bleeding complications and fibrinogen level following thrombolysis is controversial.[30] For t-PA other parameters such as template bleeding times seem to correlate better with spontaneous bleeding.[33] However it is not unreasonable to assume that high concentrations of saruplase at a level above which no further increase in efficacy can be obtained are not desirable.

In view of the need for an acute administration of saruplase any adjustment of dose based upon abnormal liver blood flow would require acute measurement or another technique to accurately identify patients with impaired liver perfusion. This is currently not feasible but this study has indicated that patients in clinical heart failure (elevated Killip classification) are likely to have reduced liver perfusion (as measured by ICG clearance) and high saruplase concentrations, but this would require confirmation in a larger study. Another useful method may be echo-Doppler measurement of hepatic blood flow[34-36] and this has been used in healthy volunteers after administering saruplase.[37] Direct measurement of saruplase concentrations by bedside kits may be the most direct approach, but such a method is presently unavailable.

Our data indicate the potential for drug-drug interactions between saruplase and drugs affecting liver blood flow either directly or indirectly by influencing cardiac output. ACE inhibitors are likely to be administered to patients with heart failure after MI after the results of recent studies.[38-40] Enalapril and captopril have been reported to decrease liver blood flow.[41,42] Glyceryl trinitrate

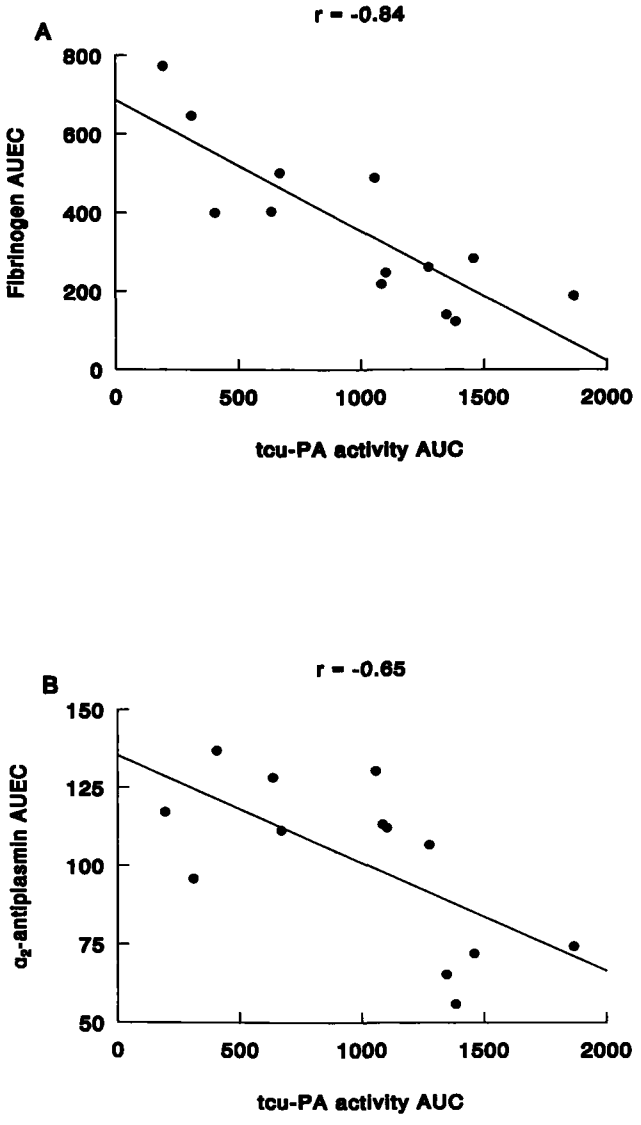

Figure 5. Relationship between fibrinogen (A) and α_2-antiplasmin levels (B) and the plasma concentration of tcu-PA activity.

also can reduce apparent liver blood flow.[43] Interactions between saruplase and these drugs (and other vasoactive medications) must be studied in MI patients rather than healthy volunteers. Drug induced changes in pre-or afterload may have quite different effects on splanchnic blood flow when cardiac performance is depressed than in the normal circulation.

The need for dose adjustment in patients with cardiogenic shock is currently unclear and requires further studies to outline the safety profile of saruplase. Consequently we do not suggest that the doses of thrombolytic therapy should be adjusted in every patient with a clinical suspicion of reduced liver perfusion. However these data may help to outline factors that influence the available amount of saruplase in the circulation and as such may lead to more optimal dosing schedules.

Acknowledgements

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CHAPTER 6

EFFECTS OF CHANGES IN LIVER BLOOD FLOW ON THE PHARMACOKINETICS OF TISSUE-TYPE PLASMINOGEN ACTIVATOR (ALTEPLASE) DURING THROMBOLYSIS IN PATIENTS WITH ACUTE MYOCARDIAL INFARCTION

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Summary

Background: *Removal from the blood of recombinant tissue-type plasminogen activator (rt-PA; alteplase) by the liver is so rapid that the liver blood flow is the rate determining step for its clearance. In patients with myocardial infarction liver blood flow may vary in response to cardiac performance and drug treatment. We estimated the effect of variations in liver blood flow in these patients on t-PA plasma concentrations during thrombolytic therapy.*

Methods and Results: *Fifteen patients with acute myocardial infarction were investigated. They received standard thrombolytic treatment with rt-PA (100 mg over 3 hours). Indocyanine green was given concurrently with rt-PA to measure liver blood flow, which was also determined by echo-Doppler. Concentrations of t-PA antigen, t-PA activity, indocyanine green, α_2-antiplasmin, fibrinogen, and*

fibrin and fibrinogen degradation products were measured. Heart rate, blood pressure, and Killip classification were used as measures of clinical condition.

Indocyanine green clearance and clearance of both t-PA antigen ($r = 0.78$; $p<0.01$) and t-PA activity ($r = 0.54$; $p<0.05$) were significantly related. Significant associations between t-PA antigen and fibrin and fibrinogen degradation products and between t-PA antigen and α_2-antiplasmin were also found.

***Conclusions:** Changes in liver blood flow of patients with myocardial infarction are inversely correlated with plasma concentrations of t-PA. In patients with severely impaired cardiac output high t-PA plasma concentrations may occur if standard doses are given. This finding may lead to optimalisation of the dosage of t-PA by interpretation of clinical conditions that represent the state of liver blood flow.*

Introduction

Recombinant tissue type plasminogen activator (rt-PA; alteplase) is a thrombolytic drug that is used extensively in patients with acute myocardial infarction.[1] In terms of patency and mortality rt-PA is comparable to other thrombolytic drugs, but its use is associated with a higher incidence of haemorrhagic strokes in comparison with streptokinase.[2] Accelerated rt-PA administration, where 100 mg rt-PA is given in 90 minutes instead of 180 minutes, provides a possible increased survival benefit compared to streptokinase, although the rate of haemorrhagic strokes remains higher.[3]

The liver exclusively and efficiently eliminates t-PA from the blood.[4] The blood flow to the liver is rate determining for the clearance of t-PA. In healthy volunteers a reduction in liver blood flow induced by exercise increased t-PA plasma concentrations proportional to the achieved reduction in blood flow.[5]

Large interindividual differences in steady state t-PA concentrations of patients with acute myocardial infarction were found after infusion of equal doses of rt-PA,[6,7] while plasma concentrations were less variable in healthy subjects.[8] After adjustment of the rt-PA dose for each patient's weight the variability was somewhat reduced but still persisted[9] and this remaining variability could be caused by the diversity in liver blood flow. An analogous finding has been reported for the high-clearance drug lidocaine. High plasma lidocaine levels and reduced clearance in patients with congestive heart failure was explained by the reduced liver blood flow in this group.[10,11]

Variability in t-PA plasma concentrations may have important clinical implications because it may determine the efficacy and side effects of t-PA to a significant extent. Indeed, coronary thrombolysis[12] and the occurrence of major bleeding in nonsurgical patients[13] are dose dependent. A reduction in the variability could theoretically lead to a reduction in serious side effects by elimination of inordinately high concentrations and an increase in efficacy in

patients who are expected to have low plasma concentrations.

We investigated the variability in liver blood flow in patients with acute myocardial infarction and the effect on plasma concentrations of t-PA by measuring liver blood flow by continuous infusion of indocyanine green. As a secondary objective we attempted to identify potential clinically useful markers for a reduction in liver blood by applying echo-Doppler measurements of liver blood flow.

Methods

Patients

Patients presenting to the hospital less than 6 hours after the onset of symptoms of acute myocardial infarction, with chest pain lasting at least 30 minutes despite the use of sublingual nitrates and accompanied by electrocardiographic signs where for anterior infarction the sum of ST-segment elevation was ≥ 0.6 mV in V1 - V6 and for inferior infarction the sum of ST-segment elevation was ≥ 0.6 mV in II, III, AVF, V5 and V6 or the sum of ST-segment elevation in II, III, AVF, V5 and V6 plus ST-segment depression in V1 - V4 ≥ 1.2mV were eligible for enrolment irrespectively of their clinical condition. The standard exclusion criteria for thrombolytic therapy were applied. Patients gave informed consent for participation, and the protocol was approved by the Hospital Ethical Committee.

Trial Design and Treatment
Medication

Patients were treated at a single center and received conventional rt-PA thrombolytic treatment with a bolus injection of 10 mg rt-PA (alteplase, Boehringer Ingelheim, Germany), followed by a continuous infusion of 50 mg over 60 minutes, and 40 mg over the next 120 minutes. A 5000 IU bolus injection of heparin was given prior to the rt-PA bolus and a 1000 IU/h heparin infusion was started as soon as possible with the dose adjusted to raise the activated partial-thromboplastin time 1.5 to 2.5 times the control and continued for 48 hours. Acetylsalicylic acid was given orally as a 250 mg initial dose and continued orally at a dose of 80 mg per day.

Measurement of liver blood flow

Simultaneously with the rt-PA infusion 90 mg of indocyanine green (Hynson, Westcott & Dunning, Baltimore, USA) was administered as a continuous infusion over 180 minutes. Indocyanine green is a tricarbocyanine dye which is

commonly used to estimate liver blood flow.[14] It is exclusively removed by the liver without biotransformation[15] and does not undergo enterohepatic circulation. The hepatic extraction ratio in man is high (≥ 0.7).[16]

Liver blood flow was measured independently by echography of the portal venous system during drug administration (every half hour when possible) using an echograph (Hewlett Packard Sonos 1500) equipped with a 3.5/2.7 MHz pulsed wave transducer. After visualisation of the portal vein or the intra-hepatic right portal vein branch the velocity spectrum was recorded. Vessel diameter and maximal blood flow velocity (Vmax) were measured from the recorded images. The cross-sectional area (CSA) of the portal vein or branch was calculated from the vessel diameter assuming a circular geometry. Blood flow volume (Q; ml/min) was calculated as: Q = CSA * Vmax.

Management

After determination of eligibility the cannulas for administration of the medication and the indocyanine green were inserted into a convenient vein of one forearm and a cannula for blood collection in the contralateral arm. Blood samples for routine haematology, biochemistry and cardiac enzymes were collected. Patients received heparin and acetyl salicylic acid and the thrombolytic therapy and indocyanine green infusion were started. Blood samples for the determination of t-PA antigen and activity were collected shortly before and 2, 6, 12, 24, 36, 48, 55, 61, 75, 89, 103, 117, 131, 145, 159, 165, 167, 170, 176, 185, 195, and 205 minutes, for indocyanine green measurements before and 12, 24, 36, 48, 55, 61, 75, 89, 103, 117, 131, 145, 159, 165, 167, 170, 173, 176, 180, and 185 minutes, and for the haemostatic parameters before and 36, 103, 165, and 225 minutes after start of the bolus injection of rt-PA. Heart rate and systolic and diastolic blood pressure were monitored before administration and 10, 30, 50, 65, 95, 125, 155, 170, 195, and 225 minutes after start of the infusion. Killip class[17] was determined upon arrival of the patient and 133, and 225 minutes after start of the thrombolytic treatment and when clinical changes of the patient were observed.

Blood Sampling

The cannula for blood collection was kept patent by continuous infusion of saline without heparin. Blood was taken after the saline infusion was interrupted and after discarding the contents of the cannula. All the samples were immediately put on ice. For the t-PA antigen measurement 4.5 ml of blood was collected in CTAD tubes (1/10 volume of 0.11 mmol/l citric acid, 15 mmol/l theophylline, 3.7 mmol/l adenosine, 0.198 mmol/l dipyridamol; Becton Dickinson, Franklin Lakes, USA). For the t-PA activity 4.5 ml of blood was collected in Stabilyte

vacutainer tubes (0.5 ml acidic citrate; Biopool, Umeå, Sweden). Blood (3 ml) for the indocyanine green assay was collected in lithium heparin tubes (Sarstedt, Nümbrecht, Germany). Determinations of fibrinogen, total fibrin and fibrinogen/fibrin degradation products, and α_2-antiplasmin were performed after collecting blood (3 ml) in 0.1 volume of 3.8% ice cold citrate. The samples contained prostaglandin E_2, theophylline and PPACK (final concentrations in blood 0.09 μM, 1 mM and 10 μM, respectively). After centrifugation of the tubes at 3000 g for ten minutes at 4°C, platelet poor plasma was collected, snap frozen and stored at -20°C until analysis.

Assays

Plasma concentrations of t-PA antigen were determined by an enzyme-linked immunosorbent assay technique[18] employed as described before.[19] For the activity assay plasma samples were acidified to neutralize inhibitors and diluted as described before,[19] using a spectrophotometric plasmin generation assay.[20] Plasma levels of indocyanine green were determined by high performance liquid chromatography according to Rappaport & Thiessen[21] in a slightly modified form using diazepam as internal standard. The α_2-antiplasmin was determined by the immediate plasmin inhibition test using H-D-Val-Leu-Lys-pNA as plasmin substrate according to an improved automated method of Friberger.[22] Fibrinogen was measured according to Clauss.[23] Prior to fibrinogen, total fibrin and fibrinogen/fibrin degradation products were measured in all samples with an enzyme-linked immunosorbent assay method that equally detects both types of degradation products.[24]

Pharmacokinetic analysis and statistics

Pharmacokinetic parameters were calculated using a standard one-compartment pharmacokinetic model for indocyanine green and a two-compartment model for t-PA (Siphar software package; Simed, Créteil, France). From the fitted functions the pharmacokinetic parameters clearance, half-life of the first exponential phase ($t½_{\lambda 1}$), half-life of the second exponential phase ($t½_{\lambda 2}$), and volume of distribution of the central compartment (Vc) were calculated according to conventional techniques. The area under the plasma concentration curve (AUC_{0-t}) was determined by means of the trapezoidal rule without extrapolation to infinity. Model independent clearance of t-PA and indocyanine green was calculated by the formula Clearance = Dose/AUC_{0-t}. The area under the effect curves of the haemostatic parameters fibrinogen, α_2-antiplasmin and total fibrin and fibrinogen/fibrin degradation products were calculated by the same method and related to the AUC_{0-t} of t-PA activity. Pearson's correlation coefficients between measurements were evaluated. Results are given as means $\pm$ SD.

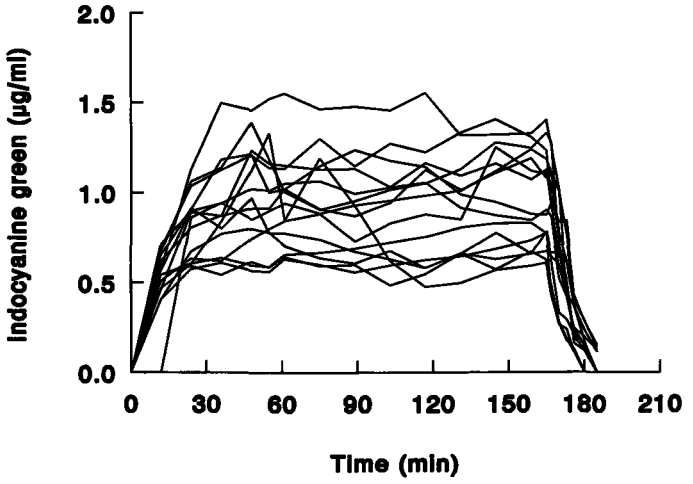

Figure 1. Individual indocyanine green plasma concentration curves of 15 acute myocardial infarct patients after infusion of 90 mg indocyanine green over 180 minutes.

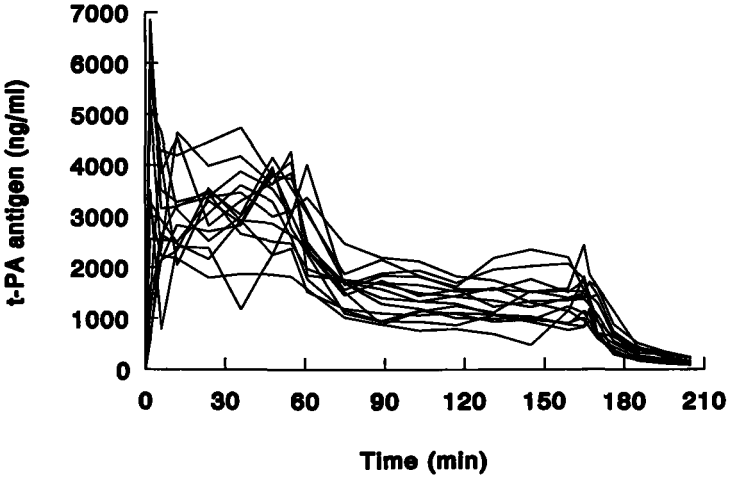

Figure 2. Individual t-PA antigen curves after a dose regimen of a bolus injection of 10 mg, followed by a continuous infusion of 50 mg over 60 minutes and 40 mg over the next 120 minutes. A similar variation can be seen for the t-PA activity concentrations.

Values were considered significantly different when $p < 0.05$.

Results

Nineteen subjects were eligible for the study. Data from two subjects were not used for analysis because of difficulties with the infusion lines during administration of the medication leading to subcutaneous infusion. Blood samples from two other patients were discarded because of failure of the storage freezer at the laboratory. The clinical characteristics of the 15 remaining analyzable patients are summarized in Table I.

Measurements of liver blood flow

The individual plasma concentration-time profiles of indocyanine green are shown in Fig. 1. Peak plasma indocyanine green levels varied from 776 ng/ml to 1553 ng/ml. The calculated clearance ranged from 366 ml/min to 809 ml/min. A similar interindividual variability was observed in clearances of t-PA antigen (Fig. 2) and t-PA activity. For t-PA antigen the clearance ranged from 207 ml/min to 421 ml/min and for t-PA activity from 304 ml/min to 816 ml/min. The pharmacokinetic parameters of indocyanine green and t-PA

Table I. Clinical characteristics (mean ± SD) of the 15 acute myocardial infarct patients.

- Sex: Male / Female	12 / 3
- Age (years)	59 ± 13
- Body weight (kg)	73 ± 11
- Infarct location: Anterior / inferior / inferolateral	8* / 3 / 4
- First / recurrent infarction	11 / 4
- Killip classification at admission: I / II / III / IV	12 / 2 / 1 / 0
- Time interval (hr) between onset of symptoms and treatment: 0-2 / 2-4 / 4-6	4 / 7 / 4
- Peak CK-MB (U/L)	123 ± 91
- Time interval (hr) between onset of symptoms and time to peak CK-MB: < 6 / 6-12 / 12 -18	2 / 8 / 5

* One patient showed the electrocardiographic signs of anterior transmural ischemia, but had no enzyme elevations after thrombolysis.

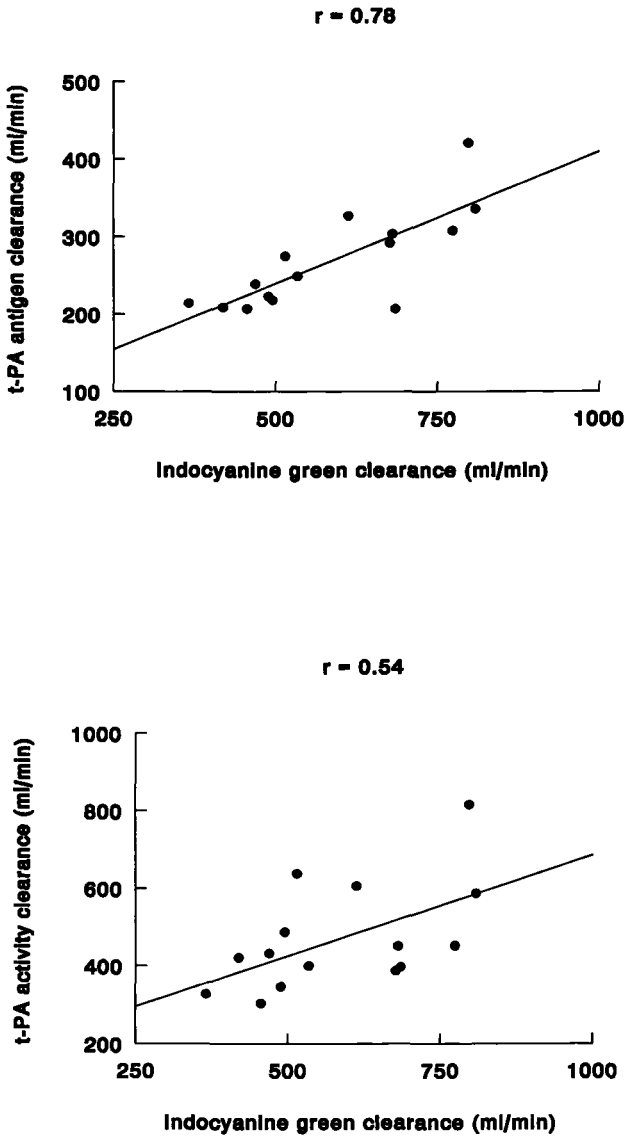

Figure 3. Significant correlations between indocyanine green clearance and t-PA antigen (upper panel) and indocyanine green clearance and t-PA activity clearance (lower panel).

derived from individual curves are displayed in Table II. A significant correlation was observed between indocyanine green clearance, as a measure for hepatic blood flow, and clearances of both t-PA antigen ($r = 0.78$; $p<0.01$) and t-PA activity ($r = 0.54$; $p<0.05$; Fig. 3).

For four subjects no echo-Doppler data were available due to the condition and/or cooperation of the patient. A significant correlation was found between indocyanine green clearance and both the right portal vein branch flow ($r = 0.63$; $n = 6$) and flow measured at the main portal vein ($r = 0.83$; $n = 5$).

Clinical observations

At admission in the hospital 12 patients were in the Killip I category and all of these patients remained in this category during the study. One patient remained in Killip class II during the entire observation period. One patient was in Killip class III at admission but recovered and was in class I at the second determination at 133 minutes after drug administration. One patient was in Killip class II at admission, slowly deteriorating to class III. After ± 4 hours she was again in the Killip II category (Fig. 4). High plasma concentrations of t-PA and the highest plasma concentrations of indocyanine green were seen in this patient.

Coagulation parameters

The fibrinogen concentration before thrombolytic treatment was 2.4 ± 0.6 g/L and total fibrin and fibrinogen degradation product concentration 0.7 ± 0.3 μg

Table II. Pharmacokinetic parameters (mean ± SD) of indocyanine green, t-PA antigen, and t-PA activity.

	Area under the curve* (units/ml*min)	Clearance* (ml/min)	Clearance† (ml/min)	Vc† (L)	$t½_{\lambda 1}$† (min)
Indocyanine green (μg)	149 ± 37	585 ± 144	539 ± 131	5.4 ± 2.1	7.0 ± 1.7
rt-PA antigen (μg)	360 ± 74	269 ± 63	291 ± 73	4.7 ± 7.3	4.2 ± 1.5
rt-PA activity (IU)	84 ± 21	470 ± 138	486 ± 124	3.8 ± 1.0	4.4 ± 1.2

* Model independent parameters
† Model dependent parameters

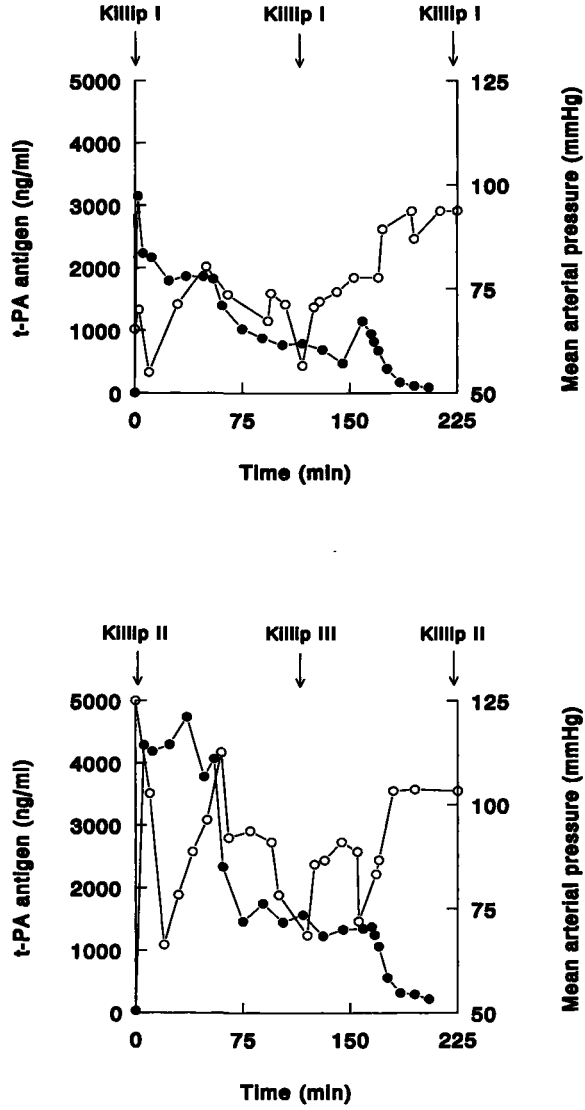

Figure 4. t-PA antigen concentrations (-●-) and mean arterial pressure (-○-) of a patient (A) who remains in Killip I category and a patient (B) with congestive heart failure (Killip II and III). Patient B received a nitroprusside infusion (60 minutes) 20 minutes after starting the thrombolytic treatment.

FE (fibrinogen equivalents)/ml. At the last measurement approximately 4 hours after drug administration the average lowest fibrinogen level of 2.0 ± 0.5 g/L and the highest total fibrin and fibrinogen degradation product level of 49.2 ± 54.9 μg FE/ml was found. No significant correlations between the area under the effect curve of fibrinogen and the area under the curve of t-PA antigen and activity were observed. The area under the effect curve of total fibrin and fibrinogen degradation products and the t-PA antigen area under the curve ($r = 0.55$; $p<0.05$) were significantly related. The functional α_2-antiplasmin decreased to 13 ± 9% of the normal value after 165 minutes. A negative correlation was seen between the area under the effect curve of α_2-antiplasmin and the t-PA antigen area under the curve ($r = -0.70$; $p<0.01$).

Discussion

This study has demonstrated that the variability in plasma concentrations of t-PA are at least partly explained by differences in liver blood flow. This correlation could be detected in a relatively small patient group that did not include patients with severely impaired cardiac function. A similar correlation between liver blood flow and clearance of lidocaine was demonstrated earlier by Zito et al.[10] in patients with congestive heart failure. A much larger group of patients with severely impaired liver blood flow was observed in their study. Although in our study no patients were in shock, the data suggest that a patient with already moderate heart failure may have impaired liver blood flow. Perfusion of vital organs may be preserved at the expense of splanchnic blood flow resulting in reduced liver perfusion while left ventricular systolic and end diastolic pressure are still in the normal range. It can be predicted that in patients in shock, even higher t-PA levels will be present than observed in this study. High t-PA antigen levels appear to be associated with the occurrence of bleeding complications[13] and the probability of having any bleeding event is related to the total dose of rt-PA administered.[19]

The relationship between measurements of t-PA activity and liver blood flow was less pronounced than for t-PA antigen. This interindividual difference is reflected in a large variety in the ratio between activity and antigen values and also indicates that liver blood flow is not the only factor determining variability.

Is it possible to clinically recognise patients with differences in liver blood flow and thereby a wide range in t-PA plasma concentrations by other more sensitive means than the Killip classification? A reliable measurement of t-PA concentrations requires complicated sample handling and frequency of measurement that cannot be implemented in the routine coronary care. Bedside determination of t-PA plasma concentrations would obviously be preferable and a new device for rapid measurements has recently become available that may offer considerable value for concentration guided dosage of rt-PA (Cardiovascular Diagnostic Inc., Raleigh, NC, USA: personal communication). Patients with

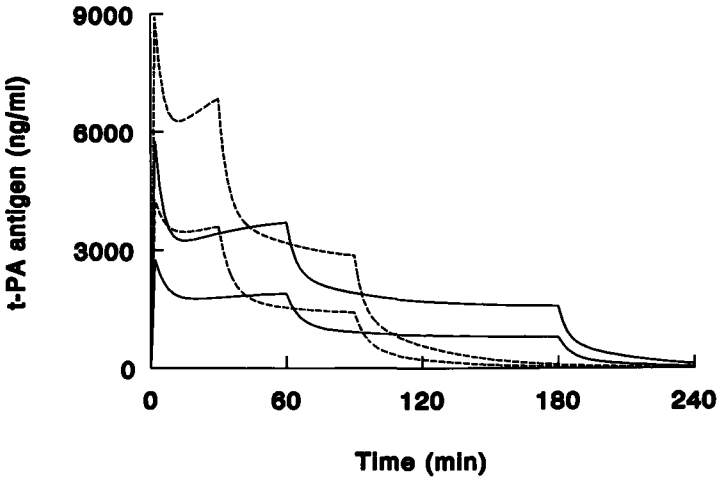

Figure 5. Comparison of simulated plasma profiles after the conventional (——) and the 'accelerated' (- - -) rt-PA dosing scheme, displaying extremes of clearance (high clearance: lower line, low clearance: upper line) as found in this study for each regimen.

abnormalities of liver blood flow have to be recognised at or during the time they receive the rt-PA infusion. The Killip classification can discriminate various levels of congestive heart failure. The measure has the advantage of being readily available but it cannot detect patients with an increased liver blood flow. Our study is to small to indicate if this method can identify those patients with increased t-PA concentrations, but it was noteworthy that the patient who deteriorated to Killip III had higher than average t-PA concentrations and the highest ICG concentrations. Echo-Doppler measurements of liver blood flow could be an alternative. Unfortunately, measurement of flow using echo-Doppler is difficult and depends on the patient's condition, habitus and cooperation. However, it can be concluded that echo-Doppler has some potential to assess liver blood flow changes during the acute phase of a myocardial infarction and its use should be evaluated further.

The haemodynamic changes induced by the infarction itself are not the only influence on liver blood flow. Concurrently with thrombolytic treatment other drugs may be administered affect liver blood flow independently and when combined with rt-PA unexpected interactions may result. For example, acute administration of nitrates[25,26] and beta-blockers[27] decrease and calcium antagonists increase liver blood flow.[28] The effect of ACE inhibitors is unknown. On theoretical grounds an increase in liver blood flow will influence the t-PA concentrations to a lesser extent than a decrease.[29] In fact, a combination of rt-PA and a stable analogue of prostacyclin (Iloprost) did not improve coronary

artery patency or left ventricular functional recovery compared with that achieved with rt-PA alone.[30] This treatment failure was suggested to be caused by an increased t-PA clearance. Extensive knowledge of possible drug interactions should be obtained in order to prevent the occurrence of sub-therapeutic or dangerously high concentrations occur.

Using the pharmacokinetic data of the patients of this study with extremes of clearance values a comparison was made between the conventional rt-PA dosing scheme (10 mg bolus, 50 mg in 1 hour, and 40 mg in the next 2 hours) and the "accelerated" rt-PA dosing scheme (15 mg bolus, 50 mg in 30 minutes and 35 mg in the next hour). This indicates that important differences between the two regimens exists (Fig. 5). The main difference in both t-PA activity and antigen concentrations occur at the start when extremely high t-PA plasma levels may occur in patients with impaired liver blood flow. The GUSTO investigators reported that "accelerated" rt-PA given with intravenous heparin provides a survival benefit over previous standard thrombolytic regimens.[3] The superiority of "accelerated" rt-PA was suggested to be related to faster recanalization of the infarct related vessel achieved by rapid administration. This may be related to the high concentrations in some patients, but could also be the cause of an excess in bleeding complications. The lack of a head to head comparison of the two rt-PA regimens in the GUSTO study leaves this as an open possibility. We pose that caution should be applied when rt-PA in standard dosages is given to patients with severely impaired liver blood flow or in combination with drugs that may affect this and suggest that dose adjustments should be considered.

Acknowlegements

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Section IV

SLOW ACTING INHIBITORS OF TISSUE-TYPE PLASMINOGEN ACTIVATOR

CHAPTER 7

ON THE ROLE OF C1-INHIBITOR AS INHIBITOR OF TISSUE-TYPE PLASMINOGEN ACTIVATOR IN HUMAN PLASMA

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Summary

An enzyme immuno assay was developed to measure complexes of tissue-type plasminogen activator (t-PA) with C1-inhibitor in order to study the role of C1-inhibitor as an inhibitor of t-PA in plasma. In vitro experiments with melanoma and recombinant t-PA learned that purified C1-inhibitor reacts with both single chain t-PA and two chain t-PA. The rate constants ranged from 3.0 to 5.2 $M^{-1}s^{-1}$. In plasma, melanoma and recombinant two chain t-PA were hardly inhibited by C1-inhibitor, in contrast to melanoma and recombinant single chain t-PA which were inhibited to the same extent by endogenous C1-inhibitor as they were by purified C1-inhibitor. In vivo, t-PA/C1-inhibitor complex could be measured in plasma in a few cases in healthy volunteers (0.62 ± 0.43 ng/ml t-PA equivalents), after exercise (0.84 ± 0.25 ng/ml t-PA equivalents) and after a desmopressin infusion (0.26 ± 0.04 ng/ml t-PA equivalents). However, t-PA/C1-inhibitor complex was found in plasma in all cases after venous occlusion (1.7 ± 0.5 ng/ml t-PA equivalents), in peritoneal fluid from patients suffering from peritoneal inflammatory disease (2.2 ± 1.3 ng/ml t-PA

equivalents) and in plasma from healthy volunteers during a t-PA infusion (27.7 ± 18.5 ng/ml t-PA equivalents at peak level). In the last case, about 8% of the infused dose of recombinant t-PA (alteplase) was inhibited by C1-inhibitor at peak level. The half-life ($t½_\beta$) of t-PA antigen in plasma was found not to be altered when t-PA was inhibited by C1-inhibitor (4.0 min and 4.2 min, respectively). Thus, in vivo, t-PA/C1-inhibitor complex is mostly present when t-PA escapes rapid liver clearance and accumulates in one place (e.g. during venous occlusion or in peritoneal fluid) or when it circulates in high concentrations (e.g. during t-PA infusion).

Introduction

Tissue-type plasminogen activator (t-PA) circulates in human blood partly as free t-PA and partly as a complex with protease inhibitors. The main inhibitor of t-PA is plasminogen activator inhibitor type-1 (PAI-1) which reacts rapidly with t-PA (second order rate constant 10^7 $M^{-1}s^{-1}$).[1] The reaction rate of other inhibitors, among which C1-inhibitor, α_2-antiplasmin and α_2-macroglobulin, with t-PA is slow (second order rate constants range from about 1 to 190 $M^{-1}s^{-1}$),[1] therefore these inhibitors do not play an important role in the regulation of t-PA activity in normal human blood. However, the contribution of this last group of inhibitors to the regulation of t-PA activity is likely to become more important when t-PA levels in plasma are elevated and PAI-1 activity is exhausted. This might be the case during thrombolytic therapy with t-PA, when t-PA activity is cleared more rapidly than t-PA antigen, which cannot be explained only by inhibition of t-PA by PAI-1.[2,3] Thus, considering this discrepancy, it would be interesting to know more about the role of protease inhibitors other than PAI-1 in the regulation of t-PA activity. It has been demonstrated that when t-PA levels are elevated, complexes of t-PA with C1-inhibitor, α_2-antiplasmin, α_2-macroglobulin and α_1-antitrypsin circulate in the blood.[4-9] These complexes however, have not been quantified, nor has the half-life ($t½$) of these complexes in blood been determined. In this study we concentrated on the role of C1-inhibitor as an inhibitor of t-PA. An enzyme-linked immunosorbent assay (ELISA) was developed for the quantification of t-PA/C1-inhibitor complexes. This assay was applied to study complex formation between t-PA and C1-inhibitor in normal plasma and to study the occurrence of t-PA/C1-inhibitor complexes in vivo under various circumstances.

Materials and Methods

Materials

C1-inhibitor was from Novabiochem AG, Läufelfingen, Switzerland; alteplase,

recombinant t-PA (rt-PA, Actilyse®, 70% single chain/30% two chain) was from Boehringer Ingelheim b.v., Alkmaar, the Netherlands; melanoma single chain t-PA (sc-t-PA) and melanoma two chain t-PA (tc-t-PA) were purified from conditioned medium from Bowes melanoma cells[10] at this institute; duteplase (rt-PA, two chain) was a gift from the Wellcome Research Laboratories, Beckenham, Kent, England. Polyclonal goat-anti-human t-PA antibodies were from Biopool AB, Umeå, Sweden; horseradish peroxidase (HRP) conjugated polyclonal rabbit-anti-human C1-inhibitor antibodies were from DAKO A/S, Glostrup, Denmark; 3,3',5,5'-tetramethylbenzidine (TMB), TMB-substrate buffer and urea peroxide were from Organon Teknika, Boxtel, the Netherlands.

Assay buffer contained 0.03 M sodium phosphate, 0.14 M sodium chloride, 0.005 M EDTA and 0.05% Tween-20, pH 7.35; TMB/peroxide substrate solution was prepared by adding urea peroxide to 10 ml of substrate buffer (0.1 M sodium acetate/citric acid, pH 6.0) to a final concentration of 1.4 g/l. 1 ml of this peroxide/substrate buffer was diluted with 9 ml demineralized water and directly before use 200 µl of a 42 mM TMB solution in dimethylsulfoxide (DMSO) were added to give TMB/peroxide substrate solution. D-Phenylalanyl-L-Prolyl-L-Arginine Chloromethyl ketone (PPACK) was from Calbiochem, La Jolla, California. H-D-Ile-Pro-Arg-pNA (S-2288) was from Kabi Diagnostica, Mölndal, Sweden.

For gel filtration experiments by use of FPLC a Superose™ 12 column (Pharmacia LKB Biotechnology, Uppsala, Sweden) was used. The buffer used for gel filtration was 0.01 M sodium citrate containing 1.6 M KSCN and 0.01% Tween-80 at pH 6.0, in order to prevent complex formation between t-PA and inhibitors during the experiment.

t-PA antigen was measured by means of an ELISA (Imulyse V1-5, Biopool AB, Umeå, Sweden).

Blood collection

For the preparation of pooled normal plasma, blood from 21 healthy volunteers was collected in citrate according to Kluft et al.[11] Plasma samples were pooled but aliquots were kept apart so individual plasma samples could also be assayed.

Blood collection for the measurement of t-PA/C1-inhibitor complex should involve addition of PPACK (final concentration 10 µM) either to the anticoagulant or immediately after blood collection.

t-PA/C1-inhibitor complex standard

A t-PA/C1-inhibitor complex standard was prepared by incubating 1 mg (9.6 nmol) purified C1-inhibitor with 500 ng (7.7 pmol) rt-PA (alteplase) in 0.1 M Tris-HCl pH 7.7, containing 0.1% (v/v) Tween-80 (final volume 1 ml)

for 19 hours at 37°C.

The presence of free t-PA in the complex standard was checked with an enzyme immuno assay using a monoclonal antibody directed against free t-PA (MAB 12-5-3, courtesy of dr. R. Bos)[12] and with a t-PA activity assay using the substrate S-2288 (detection limits 4 ng/ml and 0.1 ng/ml, respectively).

The concentration of this standard was expressed in t-PA equivalents (t-PA eq.): 500 ng/ml t-PA eq.

ELISA specific for t-PA/C1-inhibitor complex

To set up a sandwich immuno assay (ELISA) we used polyclonal anti-t-PA antibodies for the catching phase and polyclonal anti-C1-inhibitor antibodies for tagging. The optimal coating conditions for the anti-t-PA antibodies were selected from studies of coating concentrations between 1 and 15 μg/ml in 0.1 M $NaHCO_3$. A solution of 10 μg/ml was chosen because of a good response and low background absorbance. Coating of polystyrene micro-ELISA strips (Organon Teknika, Boxtel, the Netherlands) was done overnight at room temperature (RT) (100 μl solution per well). The wells were subsequently treated for three hours with a 0.1 M $NaHCO_3$ solution containing 1% (v/v) Tween-20 (150 μl/well, RT).

A standard curve of t-PA/C1-inhibitor complex was constructed by diluting the complex standard to five different concentrations (1 - 15 ng/ml) in pooled plasma and assay buffer. Standards and samples were diluted ten times in assay-buffer in the plate and incubated for two hours at room temperature.

For the detection of bound complex, a 1:5000 dilution in assay-buffer of HRP conjugated anti-C1-inhibitor antibodies was used, which gave optimal results with respect to response and background absorbance. The incubation time was two hours at room temperature (100 μl/well). Afterwards the peroxidase reaction was started by adding 100 μl of a freshly prepared TMB/peroxide substrate solution. After 30 minutes the reaction was stopped by adding 50 μl of a 2 M H_2SO_4 solution to each well. The absorbance was measured at 450 nm with a multiscan spectrophotometer (Titertek Multiscan MCC/340, MKII, Labsystems, Helsinki, Finland).

Between all steps in the assay, wells were washed three times with assay-buffer (150 μl/well).

Cross reaction in the assay by C1-inhibitor molecules not complexed to t-PA, was checked by applying purified C1-inhibitor to the assay in several concentrations (1 - 110 μg/ml). The same check was performed for free t-PA. Four t-PA preparations (alteplase, duteplase, melanoma sc-t-PA and melanoma tc-t-PA) were applied to the assay in several concentrations between 10 and 500 ng/ml.

The specificity of the assay was also shown by use of FPLC. Pooled normal plasma, spiked with 0.5 μg/ml rt-PA (incubated for 24 hours at 37°C), was

applied to a gel filtration column. Fractions were collected and total t-PA antigen as well as t-PA/C1-inhibitor complex were measured by ELISA.

To check for the influence of molecules of t-PA other than those inhibited by C1-inhibitor on the assay, rt-PA (alteplase) was added in 7 concentrations ranging from 50 to 500 ng/ml to 7 different plasma samples with t-PA/C1-inhibitor complex concentrations ranging from 3.3 to 7.5 ng/ml t-PA eq. Immediately after the addition of t-PA, the t-PA/C1-inhibitor complex concentration was measured again.

Final ELISA procedure

Polystyrene micro-ELISA strips are coated (overnight, 100 µl/well, RT) with a 10 µg/ml anti-t-PA antibody solution in 0.1 M $NaHCO_3$. Subsequently the wells are treated with a 1% (v/v) Tween-20 solution in 0.1 M $NaHCO_3$ (3 hours, 150 µl/well, RT).

After washing the wells three times with assay-buffer (150 µl/well), 90 µl of assay-buffer and 10 µl of standards and samples are added to the wells (2 hours, RT). The wells are washed again and are incubated (2 h, 100 µl/well, RT) with a 1:5000 dilution of HRP conjugated anti C1-inhibitor antibody solution in assay-buffer.

Finally, after washing the wells three times, the peroxidase reaction is started by adding 100 µl of the TMB/peroxide substrate solution and stopped after 30 minutes by adding 50 µl of a 2 M H_2SO_4 solution. Absorbance is measured at 450 nm.

Complex formation in vitro

Complex formation between t-PA and purified C1-inhibitor was studied at the physiological concentration of C1-inhibitor (1.6 µM) in 0.1 M Tris-HCl pH 7.7, containing 0.1% (v/v) Tween-80. 100 ng/ml (1.5 nM) of t-PA (alteplase, duteplase, melanoma sc-t-PA and melanoma tc-t-PA) was added to C1-inhibitor and during incubation at 37°C samples were taken at ¼, 1, 1½, 2, 4, 8 and 25 hours. The reaction was stopped by immediate freezing of the samples and later on dilution in the assay. t-PA/C1-inhibitor complex was measured in the samples by ELISA.

The initial reaction rate was calculated using the following formula: $v = k[A][R]$ or since [R] is constant under pseudo-first order conditions: $v = k'[A]$; v is the reaction rate, [A] is the molar concentration of A (in this case t-PA), [R] is the molar concentration of R (in this case C1-inhibitor), k and k' are rate constants. k' was calculated using a formula describing $t_{½}$: $t_{½} = 0.693/k'$

To be able to use these formulae, we transformed our data from concentration

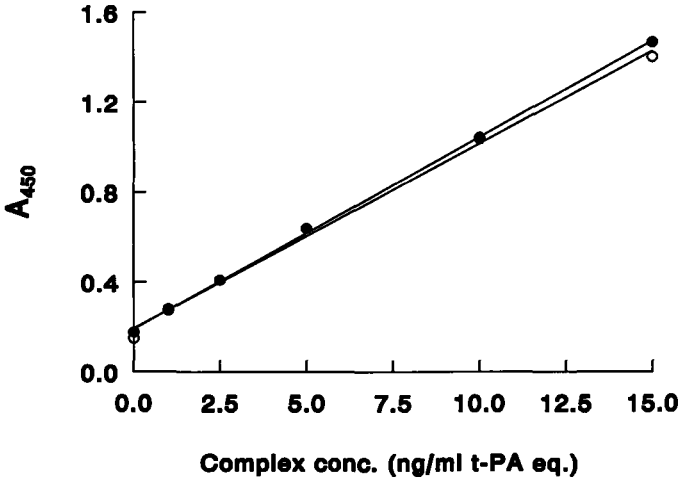

Figure 1. Typical dose-response curves for the t-PA/C1-inhibitor complex ELISA. The curves were constructed by diluting the t-PA/C1-inhibitor complex standard to five different concentrations (1, 2.5, 5, 10, 15 ng/ml) in assay-buffer (-●-) and pooled normal plasma (-O-). Curves are fitted linear.

of t-PA in complex with C1-inhibitor to concentration of free t-PA, which was possible because no other inhibitors were present during the experiment. Complex formation between t-PA and C1-inhibitor in plasma was studied similarly, adding t-PA to pooled normal plasma in several concentrations (10, 25, 50, 75 and 100 ng/ml). The interindividual variation in complex formation was studied by incubating plasma samples of 21 normal subjects with 100 ng/ml rt-PA (alteplase) for 19 hours at 37°C after which t-PA/C1-inhibitor complex was measured. The influence of the C1-inhibitor concentration in plasma on the amount of complex formed was examined by use of plasma from a patient suffering from hereditary angioneurotic edema (HANE). The HANE plasma was obtained from the 'Service central d'Immuno-Hématologie', Hôpital Lariboisière, Paris, through the courtesy of Dr J.L. Wautier. In plasma from patients with HANE, C1-inhibitor is functionally absent.[13] Pooled normal plasma was mixed with HANE plasma to C1-inhibitor concentrations of about 50% and 25% of normal plasma value. To these plasma mixtures 100 ng/ml rt-PA (alteplase) was added and t-PA/C1-inhibitor complex was measured after incubation for 19 hours at 37°C.

t-PA/C1-inhibitor complex in vivo

The occurrence of t-PA/C1-inhibitor complex in vivo was studied in various

plasma samples. Normal plasma was obtained from 21 healthy volunteers. From 5 persons, plasma samples were collected before and after venous occlusion (20 minutes). Blood was collected in ice-cold citrate. Also plasma samples were obtained from previously performed studies on the effect of exercise (J. Burggraaf, unpublished data) and desmopressin (DDAVP)[14] on fibrinolysis. Exercise (20 minutes) involved increasing heart rate to 150 beats/minute (bpm) during the first 4 minutes after which the workload of the ergometer was reduced by 25% and was kept at that level for the next 16 minutes. During these studies blood was collected in lithium-heparin (final concentration in blood 12 - 30 IU/ml) and ice-cold citrate containing prostaglandin E_2 (final concentration in blood 0.09 μM) and theophylline (final concentration in blood 1 mM), respectively. The latter was also used for blood sampling in a study in which 6 healthy volunteers were treated with a thrombolytic agent (rt-PA, alteplase, 18 mg/120 min), during which time they performed an exercise test for 20 minutes (heart rate was approximately 170 bpm at the end of the exercise period: submaximal exercise).[15] t-PA/C1-inhibitor complex was also measured in plasma and peritoneal fluid from patients suffering from pelvic inflammatory disease (PID). Blood samples and peritoneal fluid samples were collected in ice-cold citrate.

Pharmacokinetics

In case of the study involving t-PA infusion in healthy volunteers, half-lifes ($t½_{\alpha}$) of t-PA antigen and t-PA/C1-inhibitor complex in plasma were calculated using a standard two-compartment pharmacokinetic model.

Statistics

Results are expressed as mean $\pm$ SD unless stated otherwise.

Results

Sandwich ELISA for t-PA/C1-inhibitor complex
t-PA/C1-inhibitor complex standard

No free t-PA could be detected in the prepared complex standard.

Freezing and thawing of the standard showed no loss of complex in the sandwich ELISA or generation of free t-PA up till seven cycles. The standard preparation was used in the sandwich ELISA for t-PA/C1-inhibitor complex and the amount of complex was expressed in t-PA equivalents (t-PA eq.).

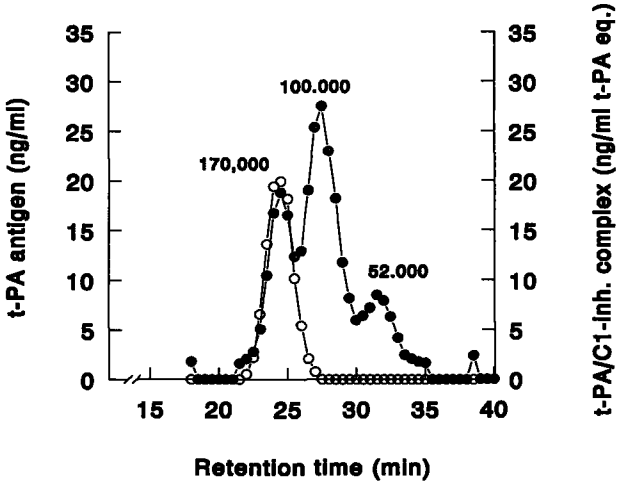

Figure 2a. Pooled normal plasma was incubated with 0.5 μg/ml rt-PA (alteplase) (24 hours at 37°C). Afterwards, the plasma was applied to a gel filtration column (by use of FPLC) and t-PA antigen (-●-) and t-PA/C1-inhibitor complex (-○-) were measured in fractions by ELISA. Buffer used for gel filtration was 0.01 M sodium citrate containing 1.6 M KSCN and 0.01% Tween-80 (pH 6.0).

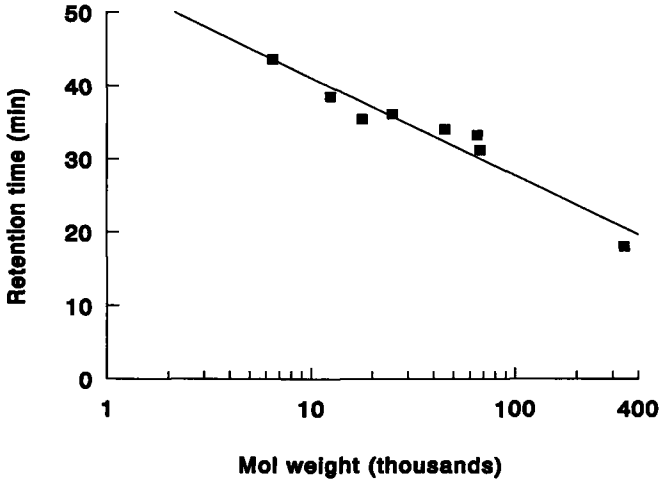

Figure 2b. Calibration curve of gel filtration column used for FLPC. Aprotinin (6.500), cytochrome C (12.400), myoglobulin (horse; 17.800), chymotrypsinogen A (25.000), ovalbumin (45.000), rt-PA (alteplase; 65.000), albumin (bovine; 67.000) and fibrinogen (340.000) were used for calibration.

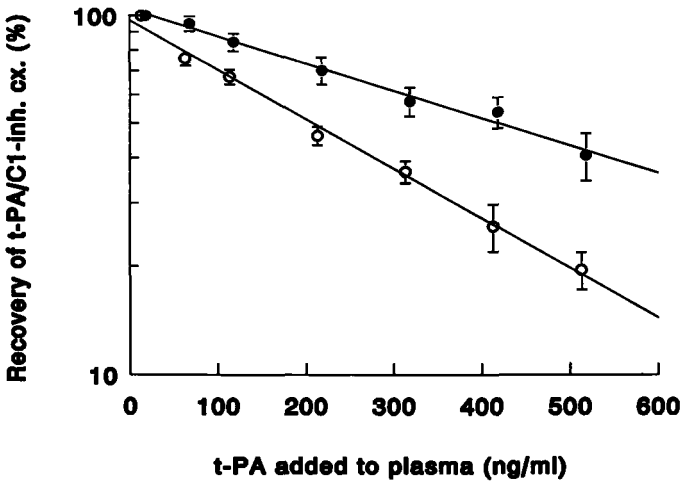

Figure 3. Influence of high concentrations of t-PA on the measurement of t-PA/C1-inhibitor complex by ELISA. To several plasma samples (n=7) containing measurable amount of t-PA/C1 inhibitor complex (range 2.9 to 7.5 ng/ml), rt-PA (alteplase; active or inactivated by PPACK) was added in increasing concentrations. Directly after adding t-PA, t-PA/C1-inhibitor complex was measured by ELISA. Results are normalised for the complex concentration measured when no t-PA was added (=100%). Data shown are means ± SEM (n=7). Curves were used for the correction of measurements of t-PA/C1-inhibitor complex in plasma, when t-PA antigen levels were higher than 50 ng/ml or 100 ng/ml in samples containing PPACK (-O-) or when PPACK was added (-●-), respectively.

Assay configuration

Measurement of dilutions between 1 and 15 ng/ml t-PA eq. of the t-PA/C1-inhibitor complex in the ELISA resulted in a linear standard curve both in pooled normal plasma and assay buffer. Fig. 1 shows both standard curves.

The reproducibility was assessed by repeated measurements on several days (n=7) in two plasma samples containing t-PA/C1-inhibitor complex (3.8 and 5.9 ng/ml t-PA eq. respectively). The inter-assay coefficient of variation was 12%. The intra-assay coefficient of variation derived from six measurements of a standard curve varied from 3 to 9% between 2.5 and 15 ng/ml t-PA eq.

Detection limit and specificity

The detection limit of the assay was calculated from 20 duplicate measurements of background absorbance in pooled plasma. For each measurement the detection limit was assessed by taking the mean background absorbance plus three times the standard deviation. For the final detection limit of the assay the mean of

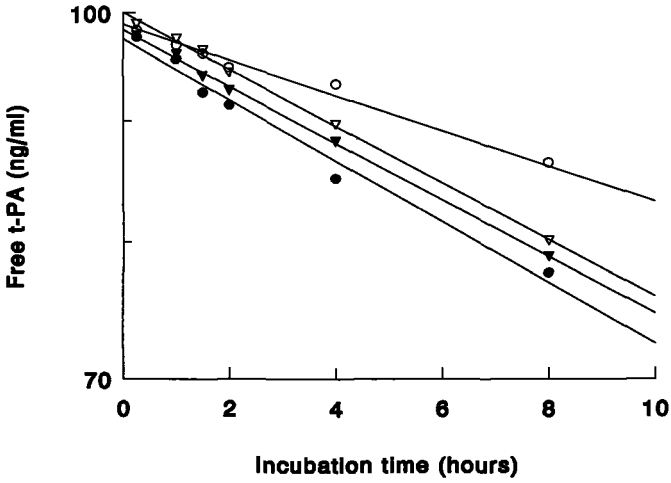

Figure 4. Inhibition of t-PA by purified C1-inhibitor. 100 ng/ml (1.5 nM) of t-PA (rt-PA; alteplase and duteplase) and melanoma t-PA (single chain and two chain) was added to 170 μg/ml (1.6 μM) of purified C1-inhibitor in 0.1 M Tris-HCl pH 7.7, containing 0.1% v/v Tween-80 and incubated at 37°C. t-PA/C1-inhibitor complex formation (ng/ml t-PA eq.) was followed in time and results were transformed to the amount of free t-PA (ng/ml). The logarithm of free t-PA is plotted against time (-▼-: alteplase, -▽-: duteplase, -●-: melanoma sc-t-PA, -○-: melanoma tc-t-PA).

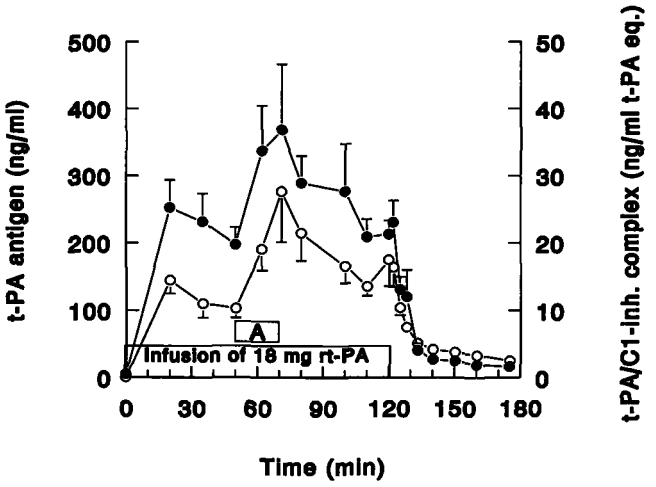

Figure 5. Infusion or rt-PA. Six healthy volunteers received a continuous infusion of 18 mg rt-PA (alteplase) over 120 minutes, during which time they performed an exercise test (A). t-PA antigen (-●-) and t-PA/C1-inhibitor complex (-○-) were measured frequently by ELISA. Data shown are means ± SEM (n=6).

these values was taken: 0.22 ng/ml t-PA eq.

No cross reaction of C1-inhibitor molecules not complexed to t-PA up to 110 μg/ml could be detected. Also four different t-PA preparations (alteplase, duteplase and melanoma sc- and tc-t-PA) showed no cross reaction in the assay in concentrations between 10 and 500 ng/ml.

The specificity of the assay as was shown by use of FPLC is shown in Fig. 2. One of the t-PA antigen peaks corresponds with the t-PA/C1-inhibitor complex peak. The retention time of this peak corresponds with a molecular weight of approximately 170.000.

We checked the influence of t-PA not inhibited by C1-inhibitor on the assay and found a decreasing response in the sandwich assay with increasing rt-PA (alteplase) concentrations, apparently due to competition between the complex and the added t-PA for the catching antibody. The results are shown in Fig. 3, normalised for the complex concentration measured when no t-PA was added (= 100%). When the same experiment was done with rt-PA (alteplase) that had been inactivated by PPACK, the influence of the free t-PA on the assay was larger (Fig. 3). Apparently, in the first experiment extra t-PA/C1-inhibitor complex was formed in vitro, probably during the two hours incubation in the microtiterplate. PPACK added to thawing plasma samples containing 100 or 400 ng/ml t-PA and a t-PA/C1-inhibitor concentration ranging from 2.4 to 5.3 ng/ml t-PA eq. had the same effect as inactivating t-PA with PPACK before addition to plasma (data not shown). We used the curves shown in Fig. 3 to correct measurements of t-PA/C1-inhibitor in plasma samples when the t-PA antigen concentration was higher than 50 ng/ml and the samples contained PPACK. When samples did not contain PPACK, measurements were corrected

Table I. Complex formation between C1-inhibitor and t-PA in plasma (citrated pooled normal plasma) after addition of 100 ng/ml of four different t-PA preparations. The amount of complex formed is expressed as the percentage of t-PA added that is complexed to C1-inhibitor.

Incubation at 37°C (h)	Alteplase (70% sc-rt-PA)	Melanoma sc-t-PA	Duteplase (tc-rt-PA)	Melanoma tc-t-PA
¼	2.4	3.6	0.8	0.5
1	8.3	10.2	1.8	1.4
1½	10.8	13.8	2.3	1.7
2	14.5	14.9	1.9	1.9
3	17.5	18.3	3.0	1.8
5	20.6	23.8	3.4	2.2
19	28.2	21.3	4.7	2.7

at t-PA antigen concentrations higher than 100 ng/ml.

Complex formation in vitro

Fig. 4 shows a representitive experiment involving the complex formation between t-PA and purified C1-inhibitor. Rate constants were calculated using the measurements up to 8 hours and were 4.8, 4.7 and 5.2 $M^{-1}s^{-1}$ for alteplase, duteplase and melanoma sc-t-PA respectively. The rate constant calculated for melanoma tc-t-PA was 3.0 $M^{-1}s^{-1}$. The experiment was performed several times and similar results were obtained. Ranges found for the rate constants are 3.8 - 5.8 $M^{-1}s^{-1}$ for alteplase (n=3), 3.9 - 4.7 $M^{-1}s^{-1}$ for duteplase (n=2), 4.0 - 5.2 $M^{-1}s^{-1}$ for melanoma sc-t-PA (n=3) and 2.8 - 3.7 $M^{-1}s^{-1}$ for melanoma tc-t-PA (n=3). In a plasma milieu a similar time-dependent complex formation between t-PA and endogenous C1-inhibitor exists, however, only for single chain t-PA preparations. Two chain t-PA formed a marginal amount of complex. Table I contains results for a dose of 100 ng/ml of t-PA. Doses of 25, 50 and 75 ng/ml showed the same time dependent pattern, while at a t-PA concentration of 10 ng/ml the influence of another inhibitor (probably PAI-1) could be seen.

The situation in plasma was similar in plasma samples from 21 healthy volunteers. A narrow range of 30.7 ± 4.2 ng/ml t-PA eq. of complex was found

Table II. In vivo occurrence of t-PA/C1-inhibitor complex.

Type of sample	n	Samples in which t-PA/C1-inhibitor complex could be measured			
		Complex conc. (ng/ml t-PA eq.)	t-PA ag. conc. (ng/ml) (range)	% of t-PA in complex (range)	n
Normal plasma	21	0.62 ± 0.43	2.1 - 5.4	4 - 49	4
Plasma after venous occlussion	5	1.7 ± 0.5	23.0 - 55.0	3 - 6	5
Plasma after exercise	7	0.84 ± 0.25	6.0 - 22.0	4 - 16	3
Plasma after DDAVP infusion	8	0.26 ± 0.04	15.3 - 16.0	1.6 - 1.8	2
Peritoneal fluid from PID patients	8	2.2 ± 1.3	27.7 - 182	0.9 - 11	8
Plasma from PID patients	8	1.9	10.0	19	1
Plasma at peak level during t-PA infusion	6	27.7 ± 18.5	60.3 - 704	6 - 12	6

when 100 ng/ml rt-PA (alteplase) was added to the plasma samples and plasma was incubated for 19 hours at 37°C.

The amount of complex formed after 19 hours (at a concentration of 100 ng/ml t-PA) was found to be dependent on C1-inhibitor concentrations in plasma. When pooled normal plasma was mixed with plasma from a patient suffering from hereditary angioneurotic edema (HANE) to C1-inhibitor concentrations of 50 and 25 percent of normal values, 25.3 ng/ml and 16.5 ng/ml of t-PA/C1-inhibitor complex was formed, respectively.

t-PA/C1-inhibitor complex in vivo

Occurrence

Measuring t-PA/C1-inhibitor complex in vivo resulted in data summarised in Table II. In plasma samples from 21 healthy volunteers taken in the morning at rest, only four samples showed values above the detection limit of the assay (0.22 ng/ml). Levels were 0.62 ± 0.43 ng/ml t-PA eq., which had no relation to the total t-PA antigen levels (values ranging from 2.1 to 5.4 ng/ml). In these samples the percentage of t-PA in complex with C1-inhibitor ranged from 4.3 to 49%.

In five healthy volunteers a venous occlusion test (20 minutes) showed a rise of complex in all cases, amounting to 1.7 ± 0.5 ng/ml t-PA eq. (before venous occlusion, complex could only be detected in one case: 0.23 ng/ml t-PA eq.). On average, t-PA antigen rose from 3 to around 40 ng/ml, the percentage of t-PA in complex after occlusion was approximately 3 - 6%.

After a 20 minutes exercise period three out of seven healthy volunteers showed detectable levels of complex around the end of exercise, amounting to 0.84 ± 0.25 ng/ml t-PA eq. (n=3), while total t-PA antigen ranged from 6.0 to 22.0 ng/ml (11.7 ± 9.0 ng/ml). The percentage of t-PA in complex ranged from 4 to 16%.

At the peak in t-PA concentration after DDAVP infusion (at t=30 minutes) only two out of eight healthy volunteers showed a rise in complex levels reaching above the detection level. The values were 0.24 and 0.29 ng/ml t-PA eq., while total t-PA antigen was at that moment 15.3 and 16.0 ng/ml respectively (percentage of t-PA in complex: 1.6 and 1.8%).

In peritoneal fluid samples from patients suffering from pelvic inflammatory disease (n=8) t-PA/C1-inhibitor complex could be measured in all samples and ranged from 0.84 to 4.6 ng/ml t-PA eq. (2.2 ± 1.3 ng/ml t-PA eq.). This represented on average 4% (range 0.9 to 11%) of the total t-PA antigen present in these fluids; no correlation between concentrations of t-PA and of complex was apparent. Only in one case a detectable complex concentration (1.9 ng/ml t-PA eq.) was measured in plasma of these patients. Both t-PA antigen and complex were present in higher concentrations in the peritoneal fluid than in the plasma.

Infusion of rt-PA

In an experiment with six healthy volunteers receiving a continuous low dose infusion of rt-PA, the behaviour of t-PA/C1-inhibitor complex was studied by frequent sampling. The volunteers performed an exercise test for 20 minutes in the middle of the infusion period.

Fig. 5 shows that the curve of the t-PA/C1-inhibitor concentration in plasma follows the same time course as the curve for total t-PA antigen concentration, with a peak at 70 minutes, just at the end of the exercise period.

The peak t-PA/C1-inhibitor concentration was 27.7 ng/ml t-PA eq. (range 7.6 to 52.6 ng/ml t-PA eq.), compared to a peak in total t-PA antigen of 369 ng/ml (range 60.3 to 704 ng/ml). This indicates that 8% (range 5.6 - 12.5%) of the circulating t-PA was complexed to C1-inhibitor at that time. Given the fact that the thrombolytic agent contained 70% sc-t-PA, this means that about 11% of the circulating sc-t-PA was complexed to C1-inhibitor, assuming the in vitro finding that tc-t-PA is not complexed to C1-inhibitor in plasma is also valid in vivo.

The alpha-phase half-life ($t½_{\alpha}$) for t-PA/C1-inhibitor complex in plasma was 4.2 min (range 1.9 - 6.5 min), which was not different from the $t½_{\alpha}$ calculated for t-PA antigen in this study: 4.0 min (range 2.1 - 6.3 min).

Discussion

When t-PA levels in plasma are elevated not only PAI-1, the fast inhibitor of t-PA, but also slow inhibitors of t-PA, like C1-inhibitor and α_2-antiplasmin, play a role in the regulation of t-PA activity.[4-9]

To quantitate this role of C1-inhibitor as an inhibitor of t-PA, we developed a sandwich ELISA to measure t-PA/C1-inhibitor complex using a polyclonal anti-t-PA antibody as catching antibody. As could be expected, excess of free t-PA in a sample influenced the assay results because t-PA competed for the catching antibody with the t-PA/C1-inhibitor complex. The influence of excess t-PA in samples containing PPACK was greater than in samples without PPACK, which indicates the in vitro formation of t-PA/C1-inhibitor complex in the latter case.

To correct for this influence we constructed a correction curve, both in the presence and the absence of PPACK, which we applied when measuring t-PA/C1-inhibitor complex in samples with t-PA concentrations higher than 50 ng/ml or 100 ng/ml respectively.

In vitro experiments with purified C1-inhibitor showed that both sc-t-PA and tc-t-PA were inhibited by C1-inhibitor. Rate constants were 4.8 and 4.7 $M^{-1}s^{-1}$ for alteplase and duteplase and 5.2 and 3.0 $M^{-1}s^{-1}$ for melanoma sc-t-PA and tc-t-PA respectively. The small difference between sc-t-PA and tc-t-PA from melanoma cells is in agreement with previously published data.[16] When t-PA was added to plasma, tc-t-PA (recombinant and melanoma) was hardly inhibited by C1-inhibitor, in contrast to sc-t-PA (recombinant and melanoma), indicating

another, probably faster route of inhibition for tc-t-PA in plasma e.g. by α_2-antiplasmin.

Measuring t-PA/C1-inhibitor complex in vivo shows that only in case of venous occlusion and in peritoneal fluid of patients suffering from peritoneal inflammatory disease (PID), t-PA/C1-inhibitor complex was detected in all cases. Also, a detectable level of complex was found in plasma samples from all six volunteers receiving a t-PA infusion. In this case, about 8% of the infused dose of rt-PA (alteplase) was inhibited by C1-inhibitor at peak level.

We therefore conclude that t-PA/C1-inhibitor complex mainly is detected in plasma when t-PA circulates at high concentrations (e.g. during thrombolytic treatment) or when t-PA escapes its dynamic pattern of release and rapid liver clearance ($t_{1/2}$ of 5 min) and accumulates in one place (e.g. during venous occlusion or in peritoneal fluid of patients suffering from PID). Occurrence of t-PA/C1-inhibitor complexes in the normal circulation thus might be partially due to extravascular movement of t-PA.

Acknowledgements

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CHAPTER 8

IN VITRO AND IN VIVO QUANTIFICATION OF COMPLEX FORMATION BETWEEN TISSUE-TYPE PLASMINOGEN ACTIVATOR AND ITS SLOW ACTING INHIBITORS C1-INHIBITOR AND α_2-ANTIPLASMIN IN HUMAN PLASMA

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Summary

In vitro and in vivo complex formation was studied between t-PA and its slow acting inhibitors. The question was addressed whether or not levels of complexed t-PA could be an explanation for the variability in steady state blood levels of t-PA between patients receiving thrombolytic treatment. Inhibition of t-PA by purified α_2-antiplasmin was investigated with a newly developed sandwich immunoassay and rate constants for alteplase, duteplase and melanoma sc- and tc-t-PA were 65 $M^{-1}s^{-1}$, 54 $M^{-1}s^{-1}$, 27 $M^{-1}s^{-1}$ and 49 $M^{-1}s^{-1}$, respectively. In plasma 31% of added t-PA was bound to α_2-antiplasmin after 8 hours at 37°C.

Complex formation was also studied by separating free from bound t-PA using gel filtration. Without pre-incubation at 37°C, 87% of t-PA that was added to plasma was free, while after 6 hours most t-PA (70%) was inhibited, mainly by C1-inhibitor and α_2-antiplasmin. Complex formation of t-PA with C1-inhibitor

and α_2-antiplasmin in patients (n=15) receiving thrombolytic treatment analyzed in a sample t=36 minutes after the start of the infusion. It was theoretically calculated that approximately 8.5% of the infused dosage, would have been complexed to both inhibitors. However, due to rapid clearance of the complexes, it comprised only an amount of 2% of the blood concentration of t-PA. Surprisingly, a part (8%) of circulating t-PA was gelfiltered at higher molecular weight, but still active. It is concluded that complex formation of t-PA does not significantly add to the observed variability in t-PA blood concentrations.

Introduction

Tissue type plasminogen activator plays an important role in the fibrinolytic pathway by converting plasminogen to plasmin.[1] Plasmin is an enzyme that dissolves the fibrin of a blood clot. Concentrations of t-PA in plasma are a resultant of production/secretion and clearance. Human endothelial cells produce t-PA and are generally thought to be the main source of t-PA in the blood.[2] Elimination of t-PA is rapid and mainly takes place in the liver.[3] Liver blood flow appears to be the rate determining step for the clearance of t-PA. The inverse correlation that has been found between liver blood flow and plasma concentrations of t-PA in patients receiving thrombolytic treatment can partly explain the large interindividual variation in steady state concentrations of t-PA antigen that occur after infusion of equal doses of rt-PA.[4] Part of this variation was already explained by the fact that the thrombolytic drug is not administered by weight.[5] We speculated that differences in clearance of various t-PA/inhibitor complexes could also have an influence on variability of t-PA concentration levels. Some of the inhibitors of t-PA that have been identified are the fast acting plasminogen activator inhibitors (PAI) 1 and 2 and the slow acting inhibitors C1-inhibitor, α_2-antiplasmin and α_1-antitrypsin.[6,7] When t-PA is present at sufficiently high levels to saturate its principal inhibitor PAI-1, for example during thrombolytic therapy, the slow acting inhibitors may become more important in binding to t-PA.[8,9]

Complex formation of t-PA with inhibitors in plasma was quantitated and the role of two important slow inhibitors (C1-inhibitor and α_2-antiplasmin) was investigated specifically. To measure t-PA/C1-inhibitor complexes a previously described enzyme-linked immunosorbent assay was used,[10] but a new assay was developed to be able to study t-PA/α_2-antiplasmin complex formation.

Furthermore, another method was used to investigate the role of the inhibitors of t-PA. Free t-PA was separated from bound t-PA by gel filtration and in vitro complex formation was studied in normal pooled plasma. In patients with myocardial infarction receiving thrombolytic treatment a sample during steady state levels of t-PA in the first infusion period was drawn and in vivo complex formation was studied by gel filtration. During the entire experiment pH was kept low (6.0) to avoid in vitro complex formation.

Materials and methods

Patients

Fifteen patients presenting to the hospital less than 6 hours after the onset of symptoms with electrocardiographic signs of an acute myocardial infarction were eligible for enrolment. The standard exclusion criteria for thrombolytic therapy could not be applied to any of the patients. Patients gave informed consent for participation, and the protocol was approved by the Hospital Ethical Committee. Patients were treated at a single centre and received a bolus injection of 10 mg rt-PA (alteplase, Boehringer Ingelheim, Germany), followed by a continuous infusion of 50 mg over 60 minutes, and 40 mg over the next 120 minutes. For administration of the medication a cannula was inserted in a convenient vein of one forearm and a cannula for blood collection in the contralateral arm. Blood samples for the determination of t-PA antigen and activity were collected shortly before and 2, 6, 12, 24, 36, 48, 55, 61, 75, 89, 103, 117, 131, 145, 159, 165, 167, 170, 176, 185, 195, and 205 minutes after start of the bolus injection of rt-PA. For determination of complex formation between t-PA and inhibitors a blood sample at 36 minutes was taken when steady state t-PA concentrations (6 - 60 min) in the first hour of infusion were reached.

Blood collection

For the preparation of pooled normal plasma, blood from 21 healthy volunteers was collected in citrate according to Kluft et al.[11] and plasma samples were pooled.

The cannula in the patients was kept patent by continuous infusion of saline without heparin. Blood was taken after the saline infusion was interrupted and after discarding the contents of the cannula. All the samples were immediately put on ice. For the t-PA antigen measurement blood was collected in CTAD tubes (0.11 mmol/l citric acid, 15 mmol/l theophylline, 3.7 mmol/l adenosine, 0.198 mmol/l dipyridamol; Becton Dickinson, Franklin Lakes, USA). For t-PA activity measurement and for determination of complex formation between t-PA and inhibitors, blood was collected in Stabilyte vacutainer tubes (acidic citrate; Biopool Umeå, Sweden). In Stabilyte plasma in vitro inhibition of t-PA by plasminogen activator inhibitor (PAI) will not occur.[12] When t-PA was added in vitro to a Stabilyte blood sample (concentration up to 1 μg/ml), no complex formation of t-PA with C1-inhibitor or α_2-antiplasmin could be detected. After centrifugation of the tubes at 3000 g for ten minutes at 4°C, platelet poor plasma was collected, snap frozen and stored at -40°C until analysis.

Materials

α_2-Antiplasmin was from Biopool AB, Umeå, Sweden; alteplase, recombinant

t-PA (rt-PA, Actilyse®, 70% single chain/30% two chain) was from Boehringer Ingelheim b.v., Alkmaar, the Netherlands; melanoma single chain t-PA (sc-t-PA) and melanoma two chain t-PA (tc-t-PA) were purified from conditioned medium from Bowes melanoma cells[13]; duteplase (rt-PA, two chain) was a gift from the Wellcome Research Laboratories, Beckenham, Kent, England. Diisopropyl fluorophosphate (DFP) was from Sigma-Aldrich, Bornhem, Belgium. D-Phenylalanyl-L-Prolyl-L-Arginine Chloromethyl ketone (PPACK) was from Calbiochem, La Jolla, California. H-D-Ile-Pro-Arg-pNA (S-2288) was from Chromogenix AB, Mölndal, Sweden. Polyclonal goat-anti-human t-PA antibodies were from Biopool AB, Umeå, Sweden; horseradish peroxidase (HRP) conjugated polyclonal rabbit-anti-human α_2-antiplasmin antibodies were from DAKO A/S, Glostrup, Denmark; 3,3',5,5'-tetramethylbenzidine (TMB), TMB-substrate buffer and urea peroxide were from Organon Teknika, Boxtel, the Netherlands.

TMB/peroxide substrate solution was prepared by adding urea peroxide to 10 ml of substrate buffer (0.1 M sodium acetate/citric acid, pH 6.0) to a final concentration of 1.4 g/l. 1 ml of this peroxide/substrate buffer was diluted with 9 ml demineralized water and directly before use 200 µl of a 42 mM TMB solution in dimethylsulfoxide (DMSO) were added to give TMB/peroxide substrate solution.

Assay for t-PA antigen and t-PA activity

Plasma concentrations of t-PA antigen were determined by an improved enzyme-linked immunosorbent assay (ELISA) technique.[5,14] For the activity assay plasma samples were acidified to neutralize inhibitors and plasmin generation was determined by a spectrometric method (stimulated indirect amydolytic activity).[5,15] After gel filtration t-PA antigen and activity were measured in the fractions and standard curves were made in column equilibration buffer.

Assay for t-PA/C1-inhibitor complex

Complex formation between t-PA and C1-inhibitor was determined in the fractions by a sandwich ELISA.[10]

ELISA specific for t-PA/α_2-antiplasmin complex

A t-PA/α_2-antiplasmin complex standard was prepared by incubating 75 µg (1.1 nmol) purified α_2-antiplasmin with 700 ng (11 pmol) rt-PA (alteplase) in 0.1 M Tris-HCl pH 7.7, containing 0.1% (v/v) Tween-80 for 72 hours at 37°C (final volume 1 ml). The presence of free t-PA in the complex standard was checked

with a t-PA activity assay using the chromogenic substrate S-2288. The concentration of the standard was expressed in t-PA equivalents (t-PA eq.).

t-PA/α_2-antiplasmin complex was measured in plasma and in buffer by use of a sandwich ELISA. For this assay flexible polyvinylchloride microtiter plates were coated overnight with a 10 μg/ml solution of polyclonal anti t-PA antibodies in 0.1 M $NaHCO_3$ (100 μl/well, room temperature [RT]). Subsequently the wells were treated with a 1% solution of Tween-20 in 0.1 M $NaHCO_3$ (3 hours, 150 μl/well, RT). Wells were washed (three times 150 μl/well) with assay buffer (phosphate buffered saline: 10 mM Na_2HPO_4, 1.6 mM KH_2PO_4, 150 mM NaCl, pH 7.5, containing 0.1% Tween-80) and standards and samples were incubated diluted 1:10 in assay buffer (2 hours, 100 μl/well, RT). A standard curve was made by diluting the complex standard to 5 different concentrations (1 to 15 ng/ml) in assay buffer or pooled normal plasma. Wells were washed again and a 1:1500 dilution of HRP conjugated anti α_2-antiplasmin antibodies was added for the detection of bound complex (2 hours, 100 μl/well, RT). Finally the peroxidase reaction was started by adding 100 μl of TMB/peroxide substrate solution and stopped after 30 minutes by adding 50 μl of 2 M H_2SO_4 solution. Absorbance was measured at 450 nm with a multiscan spectrophotometer (Titertek Multiscan MCC/340, MKII, Labsystems, Helsinki, Finland).

Cross reaction in the assay by α_2-antiplasmin molecules not complexed to t-PA was checked by applying purified α_2-antiplasmin to the assay in several concentrations (25 - 100 μg/ml). The same check was performed for free t-PA. Four t-PA preparations (alteplase, duteplase, melanoma sc-t-PA and melanoma tc-t-PA) were applied to the assay in several concentrations between 10 and 500 ng/ml.

t-PA/α_2-antiplasmin complex formation in vitro

Complex formation between purified t-PA and purified α_2-antiplasmin was studied at the physiological concentration of α_2-antiplasmin (1 μM) in 0.1 M Tris-HCl pH 7.7, containing 0.1% (v/v) Tween-80. To the α_2-antiplasmin preparation 100 ng/ml of four different t-PA preparations (alteplase, duteplase, melanoma sc-t-PA, melanoma tc-t-PA) was added and during incubation at 37°C samples were taken at ¼, 1, 1½, 2, 4, 8 and 24 hours. PPACK (final concentration 10 μM) was added to stop the reaction. In these samples t-PA/α_2-antiplasmin complex was measured by ELISA. The initial reaction rate was calculated using the following formula: $v = k[A][R]$ or since [R] is constant under pseudo first order conditions: $v = k'[A]$; v is the reaction rate, [A] is the molar concentration of A (in this case t-PA), [R] is the molar concentration of R (in this case α_2-antiplasmin), k and k' are rate constants. k' was calculated using a formula describing t½: $t½ = 0.693/k'$. To be able to use these formulae, we transformed our data from concentration of t-PA in complex with α_2-antiplasmin

to concentration of free t-PA, which was possible because no other inhibitors were present during the experiment.

The complex formation between t-PA and α_2-antiplasmin in plasma was studied similarly, using pooled normal plasma instead of purified α_2-antiplasmin.

Gel filtration

For gel filtration experiments a fast protein liquid chromatography (FPLC) Superose™-12 column was used. The column was equilibrated with 10 mM citrate, pH 6.0, containing 1.5 M KSCN and 0.01% Tween-80 at room temperature (RT). A pH of 6.0 was chosen to prevent complex formation between t-PA and inhibitors during the experiment. The applied flow rate was 0.3 ml/min and fractions of 0.2 ml were collected.

To investigate in vitro complex formation between t-PA and inhibitors in plasma, 0.5 μg/ml rt-PA (alteplase) was added to pooled normal citrated plasma and a sample of 100 μl was applied to the column directly or after incubation for 6 and 16 hours at 37°C.

To study in vivo complex formation in patients receiving rt-PA treatment for acute myocardial infarction, blood samples were collected when steady state concentrations were reached in the first hour of infusion (at 36 minutes) and 100 μl plasma was applied to the column.

Data analysis

For t-PA antigen and activity the area under the plasma concentration curve (AUC_{0-t}) was determined by means of the trapezoidal rule without extrapolation to infinity. Model independent clearance of t-PA was calculated by the formula Clearance = Dose/AUC_{0-t}. A correlation coefficient between t-PA antigen at 36 minutes and the mean t-PA antigen level at steady state (6 - 60 minutes) was determined. Correlation coefficients between t-PA clearance and t-PA/C1-inhibitor complex and t-PA/α_2-antiplasmin complex were also calculated. Pearson's correlation coefficients between measurements were evaluated by linear regression analysis and were considered significant when $p < 0.05$. Results are expressed as mean $\pm$ standard deviation (SD) unless stated otherwise.

Results

In vitro t-PA/α_2-antiplasmin complex formation

Free t-PA (6%) could still be detected in the complex standard that was prepared by incubating purified α_2-antiplasmin with rt-PA (alteplase) for 72 hours at

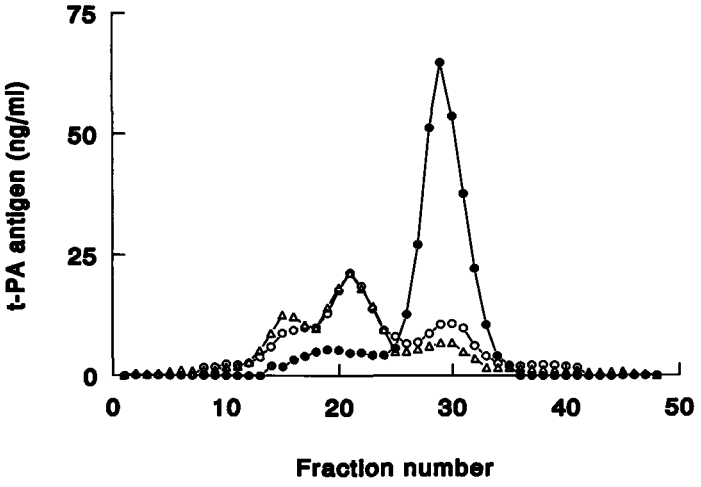

Figure 1. In vitro complex formation between t-PA and its inhibitors. To pooled normal plasma 100 ng/ml of rt-PA (alteplase) was added and gel filtration was used to separate free from bound t-PA either directly (-●-) or after incubation at 37°C for 6 (-○-) or 16 (-△-) hours and t-PA antigen was measured.

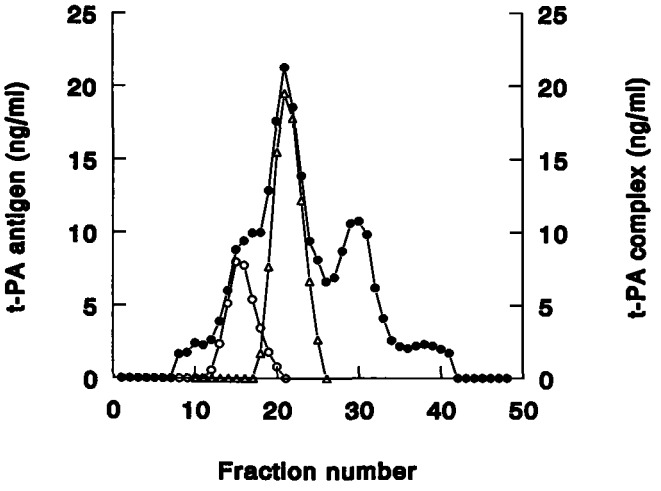

Figure 2. Identification of t-PA/inhibitor complexes present in plasma (containing 100 ng/ml rt-PA) after incubation for 6 hours at 37°C. Free and bound t-PA were separated by gel filtration and t-PA antigen (-●-), t-PA/C1-inhibitor complex (-○-) and t-PA/α_2-antiplasmin complex (-△-) were measured in the fractions using specific ELISA's.

37°C. The remaining activity was inhibited by adding 100 μM DFP. After correcting for the amount of t-PA not inhibited by α_2-antiplasmin the concentration of the standard was established as 660 ng/ml t-PA eq. Freezing and thawing of the standard showed no loss of complex in the sandwich ELISA or generation of free t-PA up till 6 cycles. The standard preparation was used in the sandwich ELISA for t-PA/α_2-antiplasmin complex to construct a standard curve. For this, dilutions were made with concentrations between 1 and 15 ng/ml in assay buffer and pooled normal plasma. Standard curves in buffer and plasma were similar.

Using the assay conditions described above, concentrations between 1 and 40 ng/ml of t-PA/α_2-antiplasmin could be adequately measured in assay buffer and plasma. The lowest concentration measured was used as the detection limit of the assay (1 ng/ml). No cross reaction of α_2-antiplasmin molecules not complexed with t-PA could be detected when purified α_2-antiplasmin was applied to the assay in concentrations up to 100 μg/ml. Also four different t-PA preparations (alteplase, duteplase and melanoma sc- and tc-PA) showed no cross reaction in the assay in concentrations between 10 and 500 ng/ml.

The complex formation between t-PA and purified α_2-antiplasmin was studied and the calculated rate constants (measurements up to 4 hours; n=2) for alteplase, duteplase and melanoma sc- and tc-t-PA were 65 $M^{-1}s^{-1}$, 54 $M^{-1}s^{-1}$, 27 $M^{-1}s^{-1}$ and 49 $M^{-1}s^{-1}$, respectively.

In plasma milieu complex formation between t-PA and endogenous α_2-antiplasmin was similar for the four different t-PA preparations. Although melanoma sc-t-PA, that had the lowest rate constant in a purified situation, was found in complex with α_2-antiplasmin for only 2.4% after 15 minutes while an average of 5.4% of alteplase, duteplase and melanoma tc-t-PA was inhibited, all preparations reached a comparable optimum level of complex formation of approximately 31% after 8 hours.

Gel filtration

An FPLC Superose™-12 column was used for gel filtration experiments. In vitro complex formation between t-PA and inhibitors in plasma was studied by adding rt-PA (alteplase) to pooled normal plasma and a sample was applied to a column either directly or after incubation at 37°C for 6 and 16 hours. Results are displayed in Fig. 1. Without pre-incubation at 37°C, free t-PA was predominantly present (87%) while only minor complex formation could be detected (13%). After 6 and 16 hours of incubation a substantial decrease in free t-PA to 31% and 24%, was found, respectively. A large part (71%) of t-PA that was not free was inhibited by C1-inhibitor and α_2-antiplasmin (Fig. 2).

In vivo complex formation of t-PA was determined in all 15 patients after having reached steady state t-PA levels at 36 minutes. The t-PA antigen levels at 36 minutes ranged from 1178 to 4738 ng/ml. These concentrations are

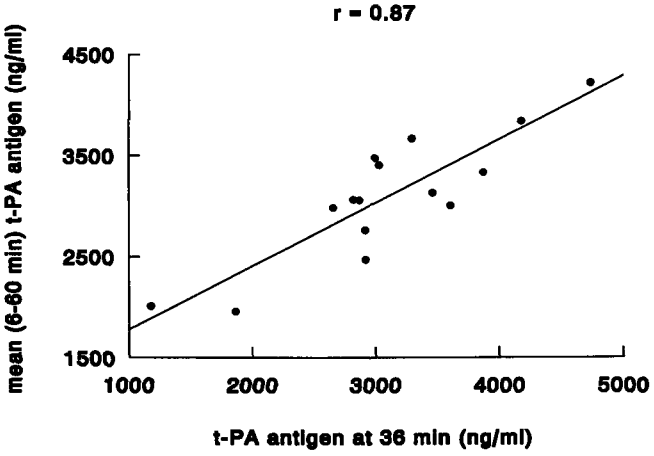

Figure 3. Correlation coefficient in 15 patients with acute myocardial infarction between t-PA antigen concentrations 36 minutes after the start of the rt-PA infusion and the average steady state antigen levels (6 - 60 minutes).

representative for the average t-PA levels at steady state (6 to 60 minutes) as shown by a significant correlation of 0.87 (Fig. 3). The t-PA levels at 36 minutes also had a positive correlation of 0.68 with the AUC_{0-t} for t-PA antigen (range: 220 to 446 μg/ml*min).

The amount of complex formation with C1-inhibitor was variable with a range of 0.8 to 4.6 ng/ml which represented only 1% of total t-PA antigen. A similar variation was found for complex formation between t-PA and α_2-antiplasmin, ranging from 0.5 to 4.3 ng/ml which also represented 1% of total t-PA antigen. Therefore, complex formation of t-PA with these two inhibitors in patients receiving thrombolytic treatment was minimal (Fig. 4). Another 8% of total t-PA antigen was also bound (molecular weight comparable to t-PA/α_2-antiplasmin complex) with a range from 9.1 to 28.0 ng/ml. However, this was still active t-PA. Levels of t-PA/C1-inhibitor complex were correlated to t-PA antigen clearance (r = -0.59). This correlation was not observed between t-PA/α_2-antiplasmin complex and t-PA antigen clearance.

Almost 90% of total t-PA in the patients was free. The variability in this free portion was large with levels ranging from 1390 to 3270 ng/ml. Concentrations of free t-PA were inversely correlated to t-PA antigen clearance (r = -0.58).

Discussion

The calculated second order rate constants for complex formation between t-PA

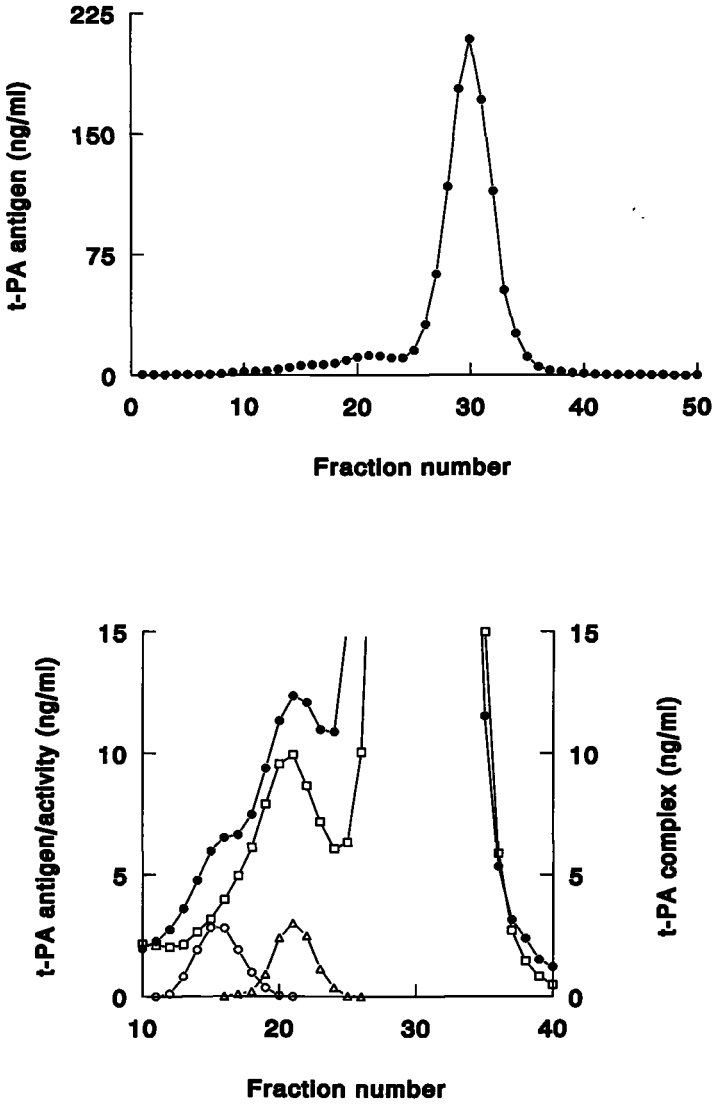

Figure 4. Plasma samples of 15 patients receiving thrombolytic therapy were fractionated by gel filtration and t-PA antigen (-●-) was measured in each fraction (upper panel). t-PA antigen (-●-), t-PA activity (-□-), t-PA/C1-inhibitor complex (-○-) and t-PA/α_2-antiplasmin complex (-△-) are presented in detail in the portion of bound t-PA (lower panel).

and purified α_2-antiplasmin were comparable to previously published data.[16] The rate constants for two-chain t-PA (melanoma tc-t-PA and duteplase) and alteplase (30% two-chain t-PA) were higher than for single-chain t-PA (melanoma sc-t-PA).

In vitro complex formation in plasma showed that approximately 70% of alteplase was bound to inhibitors after 6 hours of incubation at 37°C. As was shown in Fig. 2 a large part of the t-PA was inhibited by C1-inhibitor and α_2-antiplasmin (71% of the non-free portion). When inhibition of t-PA by α_2-antiplasmin was studied separately in plasma, 31% of t-PA was bound after 8 hours for both one- and two-chain t-PA. In a previous study complex formation between sc- and tc-t-PA and C1-inhibitor was studied and results showed that after 5 hours of incubation at 37°C ± 21% of alteplase was bound to C1-inhibitor.[10] In contrast to α_2-antiplasmin, C1-inhibitor hardly reacts with two-chain t-PA. The remaining part of approximately 20% must be bound to other inhibitors such as α_1-antitrypsin and α_2-macroglobulin.

The above described in vitro experiments can not be extrapolated simply to the clinical situation, since blood levels of complexes during use of t-PA as a thrombolytic agent are the results of formation as well as clearance of these complexes and do not reflect directly the quantity of complex that is formed.

From the kinetic data, using the average blood concentrations of t-PA it can be calculated that in the infusion time (0-36 minutes) amounts of 936 μg (= 3.1% of the dosage) and 1634 μg (= 5.4% of the dosage) of t-PA/C1-inhibitor and t-PA/α_2-antiplasmin complexes, respectively should have been formed.

The blood concentrations of complexes with both inhibitors are low, indicating rapid clearance of these complexes. Data on clearance of complexes are only known for the t-PA/C1-inhibitor complex which was shown to have a half-life closely similar to that of t-PA.[10] It can be calculated based on kinetic and clearance data that the blood concentration of the t-PA/C1-inhibitor complex should be 1% of the t-PA concentration, which agrees with the actual findings. From the blood concentrations of the t-PA/α_2-antiplasmin complexes and the kinetic data on complex formation, it is concluded that the clearance rate of the complex is also closely similar to t-PA itself. No independent data on clearance of the complex are available. The absence of a correlation of complex concentrations and t-PA clearance suggests that the t-PA moiety of the complex is not involved in the clearance. Surprisingly, a substantial part of 8% was bound t-PA according to gel filtration, but appeared to be still active. We only have information about the blood concentration at the moment of sampling and do not know the actual amount of t-PA involved in this phenomenon. The observation could be explained by a possible protein or inhibitor binding to t-PA that does not interfere with the active site or is reversible.[17] Another explanation might be the presence of a dimer of t-PA,[18] however, dimer formation was not observed in the t-PA preparation used for infusion when gelfiltered under similar conditions (data not shown).

As mentioned above, the contribution of t-PA in complex in the in vivo situation is minimal and could therefore not explain a large part of the variation in steady state concentrations of t-PA. Therefore, the large variability in t-PA antigen is probably totally due to free t-PA. The variation would be somewhat reduced after adjustment of the rt-PA dose for each patient's weight.[5] Another part of the variation is caused by the diversity in liver blood flow. The different liver blood flow values, induced by various degrees of congestive heart failure, of patients with myocardial infarction are inversely correlated with the plasma concentrations of t-PA.[4] Hereby, approximately 70% of variation in t-PA concentrations can be determined. It can be speculated that the remaining 30% partly can be explained by the possibility that part of the infused rt-PA does not remain in the intravascular compartment and is lost for several hours in the lymphatic system. Another explanation would be if a substantial part of the infused rt-PA would be bound to the endothelium. Differences between the patients in the amount of rt-PA outside the vessels or attached to the endothelium would account for variability in t-PA concentrations. However, further investigations are necessary.

Acknowledgements

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Section V

BIODISTRIBUTION OF TISSUE-TYPE PLASMINOGEN ACTIVATOR

CHAPTER 9

LOW DOSE INFUSION OF RECOMBINANT TISSUE-TYPE PLASMINOGEN ACTIVATOR (t-PA) IN HEALTHY VOLUNTEERS TO INVESTIGATE THE BIODISTRIBUTION OF t-PA IN THE EARLY PHASE OF INFUSION

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Summary

Earlier observations suggested deviations from expected pharmacokinetics of recombinant tissue-type plasminogen activator (rt-PA) in the early phase of a low dose infusion. A study with two low dose intravenous infusions of rt-PA was performed in 10 healthy volunteers in an open randomized two-way crossover design. Doses of 150 and 300 μg rt-PA were administered over 40 minutes and blood samples were collected to measure t-PA antigen concentrations. Pre- and post-infusion data were used to predict a concentration profile during infusion without t-PA binding and these values were compared to actual measurements with the t-PA binding.

*The difference between the observed and predicted area under the concentration curve over the duration of infusion was 49 ng*min/ml (95%*

*confidence interval (CI): +13,+85) for the 150 μg infusion and 29 ng*min/ml (CI: -12,+70) for the 300 μg infusion. The average amount of rt-PA that was apparently missing was 28 μg (CI: +12,+44) after the 150 μg infusion and 21 μg (CI: -75,+118) after the 300 μg infusion.*

These results indicate a distribution of t-PA over thus far unknown compartments which might include or be the endothelium. Our technique with low dose infusions is possibly valuable in defining this distribution in various patient groups and might be relevant in relation to the measurement of endothelial dysfunction.

Introduction

Tissue-type plasminogen activator (t-PA) is the primary circulating activator of plasminogen. It is synthesized and secreted by endothelial cells and it binds specifically to the endothelial surface.[1-3] In patients with angina pectoris, the level of t-PA antigen is an independent predictor of subsequent acute coronary syndromes.[4-9] Endothelial-cell injury can be a factor in the progression of coronary artery disease.

Alteplase is a recombinant form of t-PA that is used as a thrombolytic drug in cases of suspected acute myocardial infarction.[10-14] High plasma concentrations of rt-PA are necessary to obtain lysis of a clot in the coronary vessels.[15] These high concentrations can at the most for a very brief period be reached by endogenous t-PA. Extra endogenous t-PA can be released by various stimuli like histamine, adrenaline, bradykinin, thrombin and desamino-D-Arginine Vasopressin (dDAVP).[16] Exercise will also generate an increase in t-PA levels.[17,18] Antigen concentrations of approximately 25-30 ng/ml and activity levels of 5-6 IU/ml may be reached. De Boer *et al.* performed a study[19] in which a low dose intravenous infusion of 80 μg rt-PA was administered over 16 minutes that accomplished similar peak t-PA levels as was observed during exercise. A delay in appearance of rt-PA in the venous circulation was detected and the concentration profile displayed a concave rather than convex profile in the first minutes after starting the infusion. This was an indication that part of the rt-PA did not reach the systemic circulation, suggesting another unidentified compartment that might involve binding to endothelium. It appeared that this process was saturable and the possibility was raised that quantification of the amount of rt-PA involved in this phenomenon was relevant for t-PA physiology and pathophysiology. However, further study was required to explore some arbitrary mechanisms.

Firstly, in the low dose rt-PA infusion experiment of de Boer[19] 20 mg vials of sterile lyophilized alteplase powder were used. The vial contained: 20 mg alteplase, 0.7 g L-arginine, 0.2 g phosphoric acid and <1.6 mg polysorbate 80. The alteplase vial was accompanied by a 20 ml vial of sterile water to result in a 1 mg/ml concentration. For the experiment further dilution to a 20 μg/ml

concentration was accomplished with sodium chloride 0.9%. However, no extra L-arginine was added to the solution. Due to the dilution of the arginine solubilizer to less than 0.2 mg/ml it could be assumed that precipitation took place or alteplase was more easily attached to the wall of the infusion line. Moreover, the medication was not given immediately after preparation but would rest for approximately 30 minutes in the infusion line before being administered to the volunteer. This could partly explain the delay in appearance of t-PA in the venous circulation.

Secondly, in the study of the Boer[19] a very low dose of rt-PA was infused intravenously. If only a small part of the administered plasminogen activator would bind to red blood cells or other blood cells, manifestation of the drug in the venous plasma samples would be postponed and a concave profile of the plasma concentration-time curve could have been the result.

Therefore, in vitro experiments were performed with a low dose rt-PA infusion to investigate if addition of L-arginine to the solution influenced delivery from the infusion line. In addition the binding of t-PA to blood cells was investigated.

The main objective of the study was accomplished by administration of low dose rt-PA infusions in healthy volunteers. If the plasma concentration curves were to show similar concave profiles as in the experiments of de Boer[19] and the results of the in vitro experiments were to be taken into consideration, partition of t-PA to another compartment in the early phase of infusion, possibly involving endothelial binding, could be described. This technique, if feasible, could subsequently be used to study the phenomenon in pathological situations, especially in patients with endothelial dysfunction.

Materials and Methods

In Vitro Experiments

I: A solution of 1 mg/ml rt-PA (Actilyse^{R}, Boehringer Ingelheim, Germany) was diluted with 0.9% sodium chloride to a concentration of 20 μg/ml. A sample of the final solution was taken (reference sample) after which the rt-PA remained for 30 minutes in the prepared syringe and infusion line. After 30 minutes an infusion pump was started and samples of 1 ml were directly collected from the infusion line at a rate of 0.25 ml/min. The same experiment was repeated, but 0.2 M of L-arginine was added to the solution. All samples were stored at -70°C until analysis for t-PA antigen concentrations. A comparison was made between the t-PA antigen concentration-time profiles of the experiments with (N=3) and without (N=3) addition of L-arginine to the solution.

II: A solution of 1 mg/ml rt-PA (Actilyse^{R}, Boehringer Ingelheim, Germany) was diluted with 0.9% sodium chloride to a concentration of 20 μg/ml and

0.2 M L-arginine was added to the solution. A sample of the final solution was taken (reference sample) after which the rt-PA remained for 30 minutes in the prepared syringe and infusion line. After 30 minutes an infusion pump was started and samples were directly collected from the infusion line at a rate of 0.25 ml/min. The same experiment was repeated, but the solution did not remain for 30 minutes in the syringe and infusion line but the infusion pump was immediately started. All samples were stored at -70°C until analysis for t-PA antigen concentrations. A comparison was made between the t-PA antigen concentration-time profiles of the experiments with (N=2) and without (N=2) the 30 minute delay in starting the infusion pump.

III: For t-PA antigen measurements 4.5 ml of blood was collected in CTAD tubes (1/10 volume of 0.11 mmol/L citric acid, 15 mmol/L theophylline, 3.7 mmol/L adenosine, 0.198 mmol/L dipyridamol; Becton Dickinson, Franklin Lakes, USA), Stabilyte vacutainer tubes (0.5 ml acidic citrate; Biopool, Umeå, Sweden) and Monovette tubes (0.5 ml of 0.129 mmol/L sodium citrate; Sarstedt, Nümbrecht, Germany). Tubes were centrifuged at 5000 g for 6 minutes and plasma was separated from the blood cells. Concentrations of 10, 20 and 30 ng/ml of rt-PA (Actilyse^{R}, Boehringer Ingelheim, Germany) were added to the plasma (known volume) and samples were taken to measure t-PA antigen. Thereafter, blood cells were mixed with the plasma and blood was centrifuged and plasma samples were taken after 0, 5, 15, 30 and 60 minutes to measure again t-PA antigen.

For all experiments Wex Filtramed infusion lines (1.0 x 2.5 mm, length: 180 cm) were used (Wex Filtramed GmbH, Rotenburg, Germany) and Harvard syringe infusion pumps (Harvard Apparatus syringe infusion pump, model 22, South Natick, Mass., U.S.A.).

In Vivo Experiment

Subjects

A group of 10 healthy male volunteers (age 20 to 27 years, and weighing 70 to 95 kg) participated in this open randomized cross-over study after informed consent was obtained. All subjects were within 20% of their ideal weight (as defined by the Metropolitan Life Insurance Table, 1983). None of the subjects had a condition known to be associated with an increased bleeding risk (hypertension, gastro-intestinal erosions or ulcers, the use of antiplatelet drugs or acquired or congenital haemorrhagic diathesis). The volunteers were in good health as judged by medical history, physical examination, twelve-lead electrocardiogram (Nihon Kohden, Tokyo, Japan), haematology, blood chemistry, activated partial thromboplastin time, thrombin time, prothrombin time, bleeding time, fibrinogen level, and urinalysis. All test were performed within two weeks before the start of the study. None of the volunteers received

concomitant medication or recent medical treatment. The subjects had not participated in a clinical trial during the six months prior to the start of the study, had not donated blood within three months of the start of the study, did not show evidence of a recent trauma (with haematoma) or surgery in the four weeks preceding the study, and were known not to be alcohol or drug dependent. Subjects were studied after abstaining for 48 hours from alcohol and from strenuous physical activities. Caffeine containing beverages, alcohol and smoking were not allowed on a study day. The study was performed according to the principles of the "Declaration of Helsinki" (as amended in Tokyo, Venice, and Hong Kong) and the protocol was approved by the ethics committee of Leiden University Hospital.

Treatments

On one occasion an intravenous infusion of 150 μg of rt-PA (10 ml) was administered in 40 minutes. The dose was infused at a rate of 0.25 ml/min (15 μg/ml). On the second occasion an intravenous infusion of 300 μg of rt-PA (10 ml) was given in 40 minutes with an infusion rate of 0.25 ml/min (30 μg/ml). To each dose 0.2 M arginine (34.85 mg/ml) was added to prevent possible adhesion of rt-PA (Actilyse^{R}, Boehringer Ingelheim, Germany) to the infusion line. All medication was administered using syringe infusion pumps (Harvard Apparatus syringe infusion pump, model 22, South Natick, Mass., U.S.A.). An interval of one week separated the two study periods. The medication was prepared by the pharmacy of Leiden University Hospital.

Subjects arrived at the study centre after an overnight fast, beginning latest at midnight of the preceding day. The volunteers remained sitting during the entire experiment. An intravenous cannula (Venflon 18G) was inserted into a convenient vein of the forearm for administration of the medication and a second cannula was inserted into the contralateral arm for blood collection. The cannula was kept patent by continuous infusion of saline. Routine laboratory screening was repeated before drug administration on the second occasion. Before administration the volunteers rested for 90 minutes. After 40 minutes the infusion was stopped, a last blood sample was drawn, the cannulas were removed and the subjects returned home by taxi.

Blood sampling

Blood was collected after discarding the contents of the cannula. A total amount of approximately 400 ml was collected over a time period of two weeks. Samples of 4.5 ml of blood were collected in ice-cold Biopool Stabilyte vacutainer tubes (Biopool, Umeå, Sweden) containing 0.5 ml acid anticoagulant in which the blood was immediately mixed and brought to pH 6.0 in order to

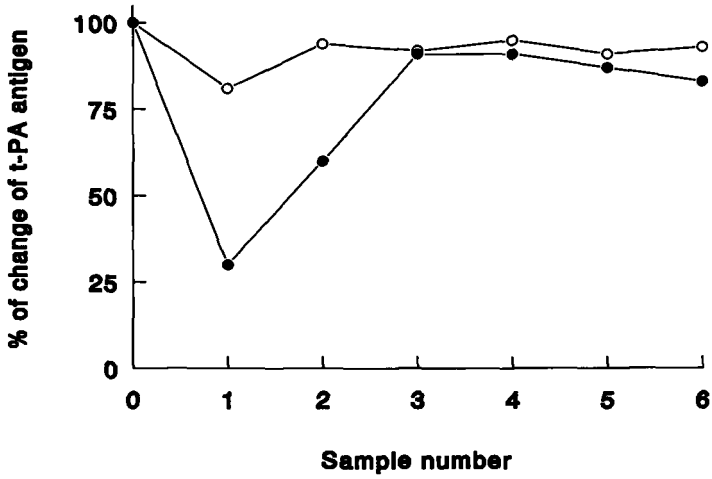

Figure 1. Mean (N=3) % of change of t-PA antigen after infusion (0.25 ml/min) of a 20 μg/ml rt-PA solution with (-○-) or without (-●-) the addition of 0.2 M L-arginine to the solution. Samples of 4 minutes were collected.

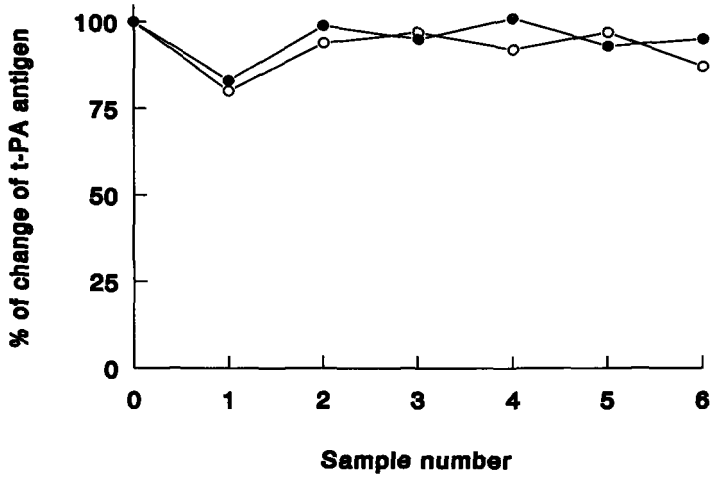

Figure 2. Mean (N=2) % of change of t-PA antigen after infusion (0.25 ml/min) of a 20 μg/ml rt-PA solution. Samples of 4 minutes were collected. The infusion pump was immediately started after preparation of the solution (-●-) or after 30 minutes (-○-).

stabilize t-PA activity.[20] Samples were taken shortly before and 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 13, 16, 19, 22, 25, 28, 31, 34, 37, 40, 41, 43, 45, 47, 49, 51, 54, 57, 60, 63, 66 and 69 minutes after drug administration. After centrifugation at 5000 g for 6 minutes at 4°C. platelet poor plasma was collected and subsequently snap-frozen and stored at -70°C.

Assays

Plasma concentrations of t-PA antigen were determined by a modified improved t-PA antigen enzyme linked immunosorbent assay technique, that was able to measure all complex formations of t-PA.[21]

Data processing

Binding of rt-PA during infusion would result in deviations of the concentration profile. In order to obtain a prediction of the profile without rt-PA binding, only the pre- and post-infusion data were subjected to non-linear regression. Pharmacokinetic parameters were calculated using a one compartment pharmacokinetic model with an additive basal activity estimate that would account for endogenous t-PA. The duration of the infusion (T) used for calculations was 38.5 minutes and not 40 minutes, because the content of the syringe required 1.5 minutes to pass the cannula that was inserted into the forearm vein. With the estimated baseline value (Base), rate of infusion (R_0), estimated clearance (CL), estimated half-life ($t_{½}$) and elimination rate constant (k) a curve could be described for the period during infusion (Formula I) and the period after ending the infusion (Formula II).

$$I: \quad C = BASE + \left(\frac{R_0}{CL}\right) * (1 - e^{-kt})$$

$$II: \quad C = BASE + \left(\frac{R_0}{CL}\right) * (1 - e^{-kT}) * e^{-k(t-T)}$$

$$k = \frac{ln2}{t_{1/2}}$$

The resulting profile predictions during infusion were compared to actual measurements. The areas under the concentration curves during infusion

($AUC_{0-38.5}$) were calculated by linear trapezoidal rule using both the observed values as well as the predicted values (Formula I). For each occasion the 95% confidence intervals (95%CI) of the differences in observed and predicted AUC were calculated. The amount of rt-PA that "disappeared" was determined by multiplying the administered dose by 1 - observed/predicted AUC_{0-end} ratio. For each infusion rate 95%CI were calculated of the amount of lost rt-PA. Calculations were carried out with SPSS/PC+ Version 4.0.1 (SPSS Inc., Chicago, Il) and BMDP dynamic Version 7.0 (BMPD statistical software Inc., Los Angeles, Ca) computer software.

Results

In vitro results

In the first experiment two rt-PA solutions, one with and one without the addition of L-arginine, remained for 30 minutes in a prepared syringe and infusion line after which 1 ml samples were collected at a rate of 0.25 ml/min. The influence on the t-PA antigen concentration-time profile was investigated of adding extra L-arginine to the rt-PA solution. When L-arginine was not added to the solution the first sample showed t-PA concentrations that were approximately 70% lower than the reference concentration and in the second sample the concentrations were 40% lower. From the third sample on the concentrations almost returned to their original levels. Although in the experiment where L-arginine was added to the solution the first sample also showed a slight decrease in concentration in comparison with the reference sample, the t-PA antigen concentration was 51% higher compared with the experiment without the L-arginine (CI:+40%,+62%). The significant difference of 33% could still be detected in the second sample (CI:+11%,+54%) but after 3 samples no difference (1.7%) between the two experiments (CI:-20%,+24%) could be observed (Fig. 1).

In the second experiment the influence was studied of the duration of time between the preparation of the syringe and infusion line with rt-PA solution (with 0.2 M L-arginine) and the start of the infusion pump. Samples were taken either directly or 30 minutes after the preparation of the syringe and infusion line. No differences could be distinguished in the rt-PA concentration-time profile between the experiment were the infusion pump was immediately started after preparation of the medication and the experiment were the solution remained in the syringe and infusion line for 30 minutes before the pump was started (Fig. 2).

In the third experiment the binding capacity of t-PA to blood cells was investigated. A decrease in concentrations of approximately 6% could always be observed when the sample where rt-PA was added to plasma was compared with the first sample at $t=0$ after mixing the plasma with the blood cells. This was

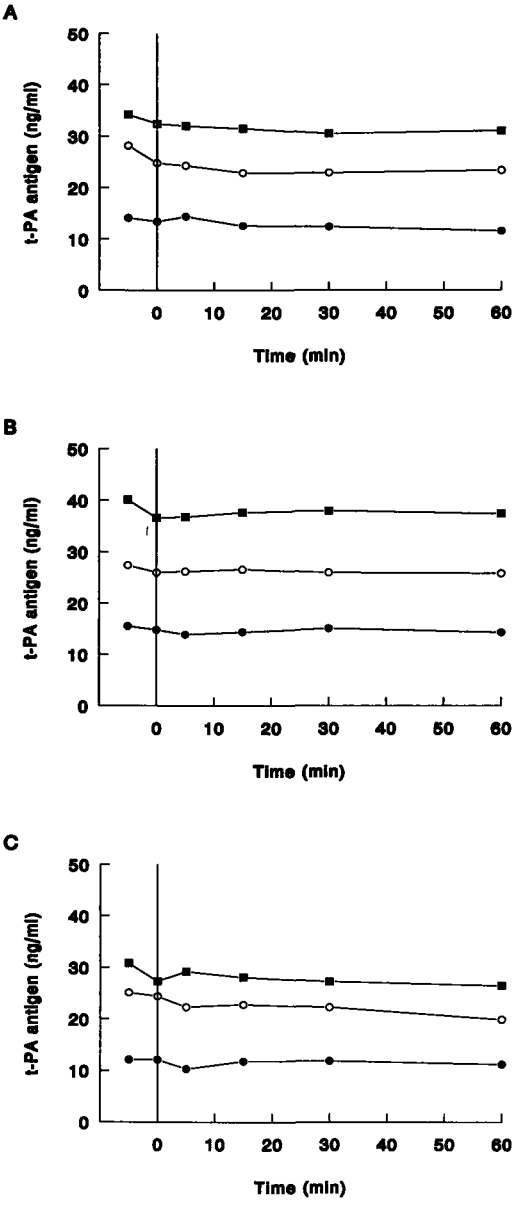

Figure 3. t-PA antigen concentrations after addition of 10 ng (-●-), 20 ng (-○-) and 30 ng (-■-) rt-PA to plasma (pre-zero values) and concentrations collected immediately and 5, 15, 30 and 60 minutes after mixing the plasma with blood cells. Tests were performed in citrated (A), Stabilyte (B) and CTAD (C) tubes.

expected, because the plasma volume becomes a comparable fraction larger after remixing it with the blood cells. All samples that were collected after mixing the plasma with the blood cells were comparable. Collecting blood in citrated, Stabilyte or CTAD tubes did not seem to be of influence (Fig. 3).

In vivo results

All subjects completed the in vivo study and during treatment with rt-PA no clinically significant adverse events were observed. A typical plasma concentration-time curve with the observed and predicted profile is presented in Fig. 4 for both occasions with either an infusion of 150 μg rt-PA (15 μg/ml) or an infusion of 300 μg rt-PA (30 μg/ml). The differences in AUC and the amount of t-PA that is apparently missing are presented in Table I. After the infusion of 150 μg the difference between the observed and predicted AUC over the first 38.5 minutes was 49 ng*min/ml (CI:+13,+85) and the difference after the 300 μg rt-PA infusion was 29 ng*min/ml (CI:-12,+70). The average amount of t-PA that "disappeared" was 28 μg (CI:+12,+44) after the 150 μg infusion and 21 μg (CI:-75,+118) after the 300 μg infusion. No difference in amount of missing

Table I. Individual differences between the observed and predicted $AUC_{0\text{-}38.5}$ and the amount of t-PA lost during infusion.

Subject	150 μg infusion of rt-PA		300 μg infusion of rt-PA	
	predicted - observed $AUC_{0\text{-}38.5}$ (ng*min/ml)	Amount of t-PA missing during infusion (μg)	predicted - observed $AUC_{0\text{-}38.5}$ (ng*min/ml)	Amount of t-PA missing during infusion (μg)
1	95.0	37.5	-14.0	0
2	86.0	67.0	60.1	102.7
3	23.4	12.5	-44.9	-34.2
4	26.5	12.5	81.7	49.7
5	20.0	9.0	27.6	14.8
6	-26.6	-10.2	50.1	30.0
7	66.1	36.4	6.9	3.2
8	51.5	40.0	87.4	54.2
9	104.2	46.2	-21.3	-40.2
10	46.8	31.5	55.3	33.5
Mean	49.3	28.2	28.9	21.4
SD	40.2	22.3	45.5	42.7

t-PA could be detected between the two different rates of administration.

Discussion

The duration of time that the solution (with addition of L-arginine) rested in the syringe and infusion line before starting the infusion pump did not influence the t-PA antigen concentration-time profile. However, the results indicate that it is essential to add extra L-arginine to a rt-PA dilution of 20 μg/ml. Without L-arginine part of the rt-PA remained in the infusion line. The total content of the infusion line was $\pm$ 1.6 ml. Therefore, the total volume of the first sample of 1 ml had been in contact with the infusion line for 30 minutes, whereas the same can be stated for only part of the volume of the second sample. The contents of the following samples were in contact with the infusion line for the six minutes it took to pass the length of the line. With the addition of L-arginine to the solution the substantial reduction in t-PA antigen concentration in the first two samples was absent. Therefore, in studies where low dose infusions are used addition of L-arginine to the final solution is necessary. The absence of extra L-arginine could partly explain the delay in appearance of t-PA in the systemic circulation of healthy volunteers and with L-arginine a possible quantification of the in vivo binding capacity of t-PA could be calculated in a more reliable way. In vitro experiments also demonstrate that the rt-PA is not lost because of binding to blood cells.

The results of the in vivo experiments illustrate that between the two rates of infusion similar values were found for either the differences in $AUC_{0\text{-}38.5}$ between the observed and predicted values or for the amount of t-PA that "disappeared". This demonstrates that the distribution of t-PA over the newly identified compartment probably did not depend on the concentration of t-PA in the blood that we achieved during infusion and is apparently saturable at these concentrations. The capacity of this compartment also appears limited and to involve around 25 μg of rt-PA. This limited capacity explains why we observed the phenomenon only in relation to experiments with infusions of low dosages of rt-PA.

The intriguing question is the identity of the newly found compartment. The possibility exist that the missing t-PA does not remain in the intravascular compartment and is lost in the lymphatic system where it may stay for several hours before returning in the blood stream. It must be assumed that the return takes place slowly and after our experimental period. It is of importance to emphasize that our model of calculating the missing portion assumes that steady state concentrations of t-PA have been reached 40 minutes after the start of the infusion. If steady state was not reached and release of t-PA from the putative new compartment would have taken place immediately after the end of infusion, the calculation of the estimated pharmacokinetic parameters, using pre- and post-infusion concentrations, would have resulted in a predictive curve almost

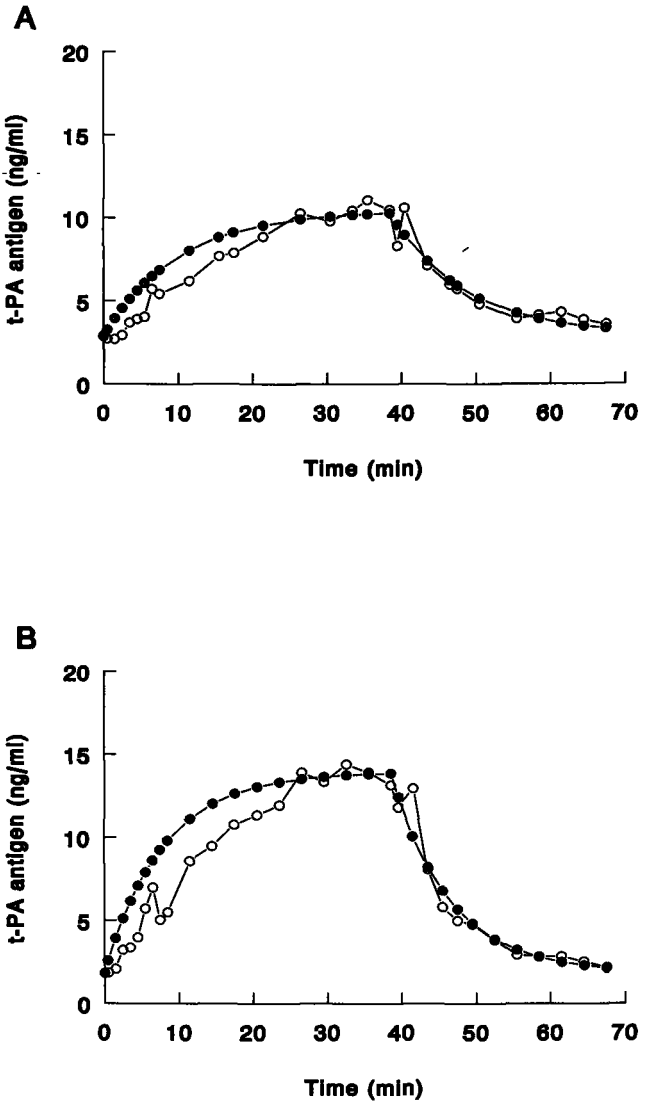

Figure 4. The observed (-○-) and predicted (-●-) t-PA antigen concentrations after infusion of 150 μg (A) and 300 μg (B) of rt-PA.

identical to the observed curve. This further characterizes the phenomenon and emphasises the importance of the detailed analysis of the post-infusion period. It indicates that the returnal of the missing portion to the circulation (if ever) is at least delayed to beyond our observation period. A second low dose infusion, that would start immediately after the first one when concentrations of t-PA would have reached pre-dose levels, should result in a concentration-time curve similar to the predicted curve of the first infusion. The extra compartment would have been 'filled' during the first infusion, and therefore, during the second infusion no additional loss of t-PA should occur. This experiment does require extensive blood sampling and was not performed in this volunteer group.

A temporary increase in liver blood flow at the time of rt-PA administration would result in higher clearance values and lower concentrations of t-PA. However, there were no indications that haemodynamic changes or the infusion itself could have induced an increase in liver blood flow. Furthermore, the moderate circadian variation in liver blood flow that has been reported indicate a decreasing flow during the morning, which would indicate an increase in t-PA rather than a decrease.

Another assumption might be that t-PA forms a complex with PAI-1 and that this complex is behaving in a different way compared to free t-PA. The 'missing' amount of 25 μg t-PA would be able to complex on average 25% of the circulating active PAI-1 in healthy volunteers. That this contributes to the phenomenon is not expected since we used a method for t-PA antigen measurement that also measures t-PA adequately in this complex.[21] In addition, it has been reported that, compared to the clearance of t-PA, the clearance of t-PA/PAI-1 complex is slower[22] and would cause an increase in recorded t-PA molar concentration rather than the observed decrease. It can be expected that an effect of t-PA/PAI-1 complex formation results in underestimation of the missing portion of t-PA. In further analysis of the phenomenon the analysis of t-PA/PAI-1 clearance should be considered, requiring specific methodology.

An intriguing possibility is the binding of t-PA to the vessel wall either to PAI-1 or endothelial binding sites.[23,24] Sites with different affinity have been described from studies on cultured endothelial cells, ranging from low affinity sites with a possible in vivo capacity of 50-500 mg t-PA, to high affinity sites with a predicted 50% occupation and additional capacity of around 100 μg t-PA.[24] These capacities have been calculated assuming a similar binding of t-PA to the different endothelial cells of the body and are only illustrating theoretical binding capacity. In our case it illustrates that in vitro data and simple extrapolation to in- vivo conditions do not refute the possibility of a high affinity low capacity binding compartment of endothelial cells.

In recent years t-PA and PAI-1 have been identified as risk markers for cardiovascular disease.[4] A relationship was identified between insulin sensitivity and an increase in both t-PA and PAI-1.[25] The increases in haemostatic parameters may also be caused by chronic inflammation, which may occur in

patients that have developed clinical or sub-clinical atherosclerosis. Changes in endothelial function or endothelial dysfunction such as reported in patients with insulin resistance or chronic vascular inflammation may play a role in the pathogenesis. However, the exact underlying mechanism is not known. Endothelial changes other than through t-PA and u-PA binding or release could be involved or changes in clearance of t-PA without any involvement of endothelial function. Our observations of a new t-PA binding compartment might concern binding to the endothelium in vivo. Among studies to further identify and characterize the compartment, studies with low dose infusions of t-PA in selected patient groups with endothelial dysfunction can be suggested to further describe variability in this compartment and its possible relation to disease and disease risk, and endothelial dysfunction.

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Section VI

CONCLUSIONS

CHAPTER 10

CONCLUSIONS AND FINAL CONSIDERATIONS

The general objective of the studies described in this study was to investigate different factors that could have an influence on the large variability in steady state plasma concentrations of high clearance thrombolytic drugs in patients with an acute myocardial infarction receiving equal dose regimens.

The role of changes in liver blood flow on the pharmacokinetics and pharmacodynamics of tissue-type plasminogen activator (t-PA) and single chain urokinase-type plasminogen activator (scu-PA) was investigated in healthy volunteers. In patients with an acute myocardial infarction the correlation between liver blood flow and steady state concentrations of the applied thrombolytic drug was studied. Standard dosages of both t-PA and scu-PA were used. Subsequently the role of two slow acting inhibitors of t-PA, C1-inhibitor and α_2-antiplasmin, was investigated. In vitro and in vivo complex formation was studied to determine the amount of t-PA/C1-inhibitor and t-PA/α_2-antiplasmin complex formation and to define the pharmacokinetic properties. Finally, the adsorption of t-PA in an unidentified compartment, possibly the endothelium, was studied.

Studies in healthy volunteers

Decreased liver blood flow, induced by a standardized exercise test, resulted in an increase in plasma concentrations of total u-PA antigen. This clear reaction indicated the importance of the liver to eliminate scu-PA from the body. However, other organs may still be involved or its metabolism in the liver may be different from that of t-PA, because other studies indicated a more prominent reaction of t-PA after an exercise test. Unexpected results were observed after the intake of food. A standardized meal clearly resulted in an increased liver blood flow, but this was not followed by decreased u-PA concentrations. This may have been caused by competition for the receptor responsible for the mediation of the internalization and degradation of pro-urokinase in the liver by a food component. The identification of this component may lead to a better understanding of the metabolism of scu-PA.

Increased liver blood flow, again induced by food, did result in the expected reduction in t-PA plasma concentrations. In view of the effect of food on u-PA levels, it is highly probable that different regulatory mechanisms exist for u-PA and t-PA, although the effect could have been caused by the administration of

unglycosylated scu-PA instead of the native glycosylated form. This study also showed that an increase in liver blood flow had a more pronounced effect on t-PA pharmacokinetics than a decrease. During the infusion of rt-PA, changes could be observed in endogenous urokinase-type plasminogen activator. More scu-PA is activated to tcu-PA resulting in decreased scu-PA concentrations and increased tcu-PA concentrations. Total u-PA antigen levels remain unchanged.

Studies in patients with acute myocardial infarction

In patients receiving thrombolytic treatment with scu-PA an inverse correlation was found between liver blood flow and u-PA antigen and activity levels. A severe reduction in blood flow caused by heart failure would lead to an increase in plasma concentrations. In this study tcu-PA activity levels were associated with systemic fibrinogenolysis, but although the correlation between bleeding complications and fibrinogen levels is controversial, a situation with high u-PA concentrations at a level above which no further increase in efficacy would be obtained is probably not desirable.

In the patients that received rt-PA it was demonstrated that the variability in plasma concentrations of t-PA could, at least partly, be explained by differences in liver blood flow. Although no study has clearly demonstrated a correlation between major bleeding complications and levels of fibrinogen or α_2-antiplasmin, high t-PA antigen levels appear to be associated with the occurrence of bleeding complications and the probability of having any bleeding event is related to the total dose of rt-PA administered.

The recognition of patients with severely impaired liver blood flow is important. Determining liver blood flow by Doppler echography seems promising. Direct bedside measurements of thrombolytic drug concentrations could lead to concentration guided dosage of both rt-PA and scu-PA and techniques for this are being developed. The use of other medication during thrombolytic treatment that could have an influence on the pharmacokinetics of t-PA or scu-PA and the haemodynamic changes induced, demand an extensive study of possible drug interactions.

Slow inhibitors of t-PA

In vivo measurements showed that t-PA/C1-inhibitor complex levels could mainly be detected when t-PA would circulate in high concentrations, that is during thrombolytic treatment, or when t-PA escapes rapid liver clearance and accumulates in one place as is the case during venous occlusion or in peritoneal fluid. The half-life of t-PA antigen in plasma was found not to be altered when t-PA was inhibited by C1-inhibitor. Therefore, even if different levels of t-PA/C1-inhibitor complex would exist between patients that receive

thrombolytic treatment, this would not explain a large variation in t-PA antigen levels between the patients.

Complex formation of t-PA with C1-inhibitor and α_2-antiplasmin in patients receiving thrombolytic treatment should theoretically have been approximately 8.5% of the infused dosage. However, 36 minutes after the start of the infusion it comprised only an amount of 2% of the blood concentration of t-PA. Both complexes are therefore rapidly cleared and it was concluded that the half-life of the t-PA/α_2-antiplasmin complex is also closely similar to t-PA itself. It could be concluded that this complex does not add to the observed variability in t-PA blood concentrations either. Further investigations to identify the unknown protein or inhibitor bound to t-PA (8%) that was found after gel filtration are required.

Biodistribution of t-PA

In the early phase of a low dose infusion of rt-PA, part of the t-PA did indeed disappear. The results indicate that t-PA is bound in thus far unknown compartments, which might include or be the endothelium. If the endothelium would be involved, further investigations should be performed in various patient groups to see if this method could give an indication of different levels of endothelial dysfunction. Impairment of the endothelial function could alter the clearance of both scu-PA and t-PA and thereby perhaps have an influence on the observed variability in t-PA blood concentrations.

SAMENVATTING

Hoofdstuk 1

Dit hoofdstuk beschrijft het gebruik van stolseloplossende middelen voor het behandelen van patiënten met een acuut myocardinfarct. In 1959 werd voor het eerst in patiënten het fibrinolytische geneesmiddel streptokinase gebruikt nadat eerder theoretische bewijzen waren geleverd voor het werkingsmechanisme van trombolyse. Het was echter pas in 1983 dat het belang van trombolyse werd erkend nadat gevonden was dat coronaire trombose chronisch coronair lijden omzette in een acuut myocardinfarct.

De fibrinolytische geneesmiddelen streptokinase, anistreplase, urokinase, weefsel-type plasminogeen activator (t-PA) en pro-urokinase (scu-PA) worden daarna besproken. De farmacokinetische en farmacodynamische kenmerken worden behandeld, de voor en nadelen van elk middel en waar ze precies aangrijpen in het fibrinolytische systeem. Het meest ideale middel leek te zijn gevonden met de introductie van scu-PA en t-PA. Deze activatoren van de fibrinolyse, en dan vooral t-PA, schenen een belangrijk voordeel te hebben boven de andere middelen door hun stolsel selectieve werking. Veel klinische onderzoeken werden met t-PA uitgevoerd om het juiste doseringsschema te vinden waarbij deze positieve kenmerken optimaal tot uiting zouden komen. De verschillen tussen streptokinase en t-PA wat betreft mortaliteit, het optreden van een tweede infarct en vooral het voorkomen van (hersen) bloedingen bleken echter minimaal te zijn.

Ook al zal ooit de ideale dosering van t-PA gevonden worden, toch wijst het meeste onderzoek erop dat er altijd patiënten zijn, waarbij reperfusie niet zal optreden of waarbij ernstige bijwerkingen gevonden zullen worden. Dit zal waarschijnlijk niet aan de dosering liggen. Het blijkt dat bij het toedienen van gelijke doseringen t-PA aan patiënten er een grotere variabiliteit optreedt in plasma concentraties van het geneesmiddel dan in gezonde vrijwilligers. Door een grote variabiliteit zullen er mensen zijn met plasma concentraties die buiten de therapeutische gebied liggen. Dit zou een verklaring kunnen zijn voor de soms teleurstellende resultaten van t-PA wanneer het middel met andere trombolytische geneesmiddelen zoals streptokinase wordt vergeleken. Het is daarom belangrijk factoren te onderzoeken die een rol kunnen spelen bij het ontstaan van deze variabiliteit.

In het laatste deel van dit hoofdstuk worden verschillende mogelijkheden voor verhoogde variabiliteit naast de effecten van lichaamsgewicht besproken. Ten eerste wordt de theoretische invloed van de leverbloeddoorstroming besproken. Zowel scu-PA als t-PA zijn middelen waarvan de eliminatie uit het lichaam afhankelijk is van de bloeddoorstroming door het orgaan van eliminatie, de

lever. Na een myocardinfarct zouden veranderingen in de hemodynamische toestand kunnen bijdragen aan een verhoogde variabiliteit in concentraties. Als tweede mogelijkheid wordt de invloed van de verschillende remmers van t-PA in het bloed besproken. Als na toediening van een therapeutische dosis t-PA aan patiënten een substantieel deel van het t-PA gebonden is, dan zou er door een andere klaring van gebonden t-PA in vergelijking met de klaring van vrij t-PA, een variatie in concentraties kunnen ontstaan afhankelijk van de mate van t-PA inhibitie per patiënt. Als laatste wordt de mogelijke rol van het endotheel besproken. Het endotheel produceert zowel endogeen t-PA als u-PA en bij het reguleren van de functies van deze twee middelen spelen specifieke bindingsplaatsen op dit endotheel een rol. De mate van aantasting van dit endotheel per patiënt zou tot variabiliteit in plasma concentraties kunnen leiden.

Hoofdstuk 2

Hierin wordt een overzicht gegeven van de verschillende doelstellingen van de onderzoeken die beschreven zijn in de hoofdstukken 3 t/m 9. Tevens worden de methoden besproken die gebruikt zijn om de uitvoer van de studies mogelijk te maken.

Hoofdstuk 3

Deze studie beschrijft de invloed van veranderingen van leverbloeddoorstroming op de farmacokinetiek en farmacodynamiek van enkelketenig urokinase-type plasminogeen activator (scu-PA).

In deze open, gerandomiseerde studie kregen 10 gezonde mannelijke vrijwilligers elk een infuus van 37.5 mg recombinant scu-PA (saruplase) en 90 mg indocyanine groen. Beide middelen werden tegelijkertijd toegediend over een periode van 150 minuten. Indocyanine groen is een kleurstof die volledig door de lever uit het lichaam wordt geklaard en is een algemeen gebruikt middel dat dient als maat voor de leverbloeddoorstroming. Op de ene studiedag werd 60 minuten na het starten van de infusen een gestandaardiseerde maaltijd (vet en eiwit rijk) genuttigd om een toename in de leverbloeddoorstroming te bewerkstelligen. Op de andere studiedag werd er 60 minuten na het starten van de medicatie een fietsproef afgelegd waardoor er een daling in de bloeddoorstroming van de lever zou moeten ontstaan. Indocyanine groen concentraties, totaal urokinase-type plasminogeen activator (u-PA) antigeen, tweeketenig u-PA activiteit, fibrinogeen, afbraakprodukten van fibrine en fibrinogeen, α_2-antiplasmine en factor XII-afhankelijke fibrinolytische activiteit werden gedurende het onderzoek gemeten. De bloeddoorstroming van de lever werd op die dagen waarop de gestandaardiseerde maaltijd werd gegeven eveneens gemeten door middel van Doppler echografie van een tak van de poortader.

De gemiddelde indocyanine groen concentratie na de fietsproef was significant verhoogd met 29% vergeleken met de basale waarden. Na voedsel waren de concentraties gemiddeld 27% lager en was de bloeddoorstroming door de poortader maximaal met 103% verhoogd. De gemiddelde maximale u-PA concentraties waren na de inspanningsproef significant met 130 ng/ml verhoogd en er was onverwacht ook een verhoging van de maximale concentraties met 156 ng/ml na voedsel. Deze verhogingen gingen vergezeld met niet significante stijgingen van gemiddelde u-PA antigeen concentraties voor zowel de inspanningstest (7%) als voor voedsel (13%). Het effect na voedsel in vergelijking met de inspanningstest werd teruggezien in kleine veranderingen in de farmacodynamische (stollings) parameters.

Uit deze studie kan geconcludeerd worden dat u-PA plasma concentraties verhoogd worden door een verlaagde bloeddoorstroming van de lever geïnduceerd door inspanning. Voedsel inname produceert een onverwachte verhoging in u-PA concentraties ondanks een toename van de bloeddoorstroming van de lever.

Hoofdstuk 4

Het doel van dit onderzoek was het effect meten van verhoogde leverbloeddoorstroming op de farmacokinetiek en farmacodynamiek van recombinant weefsel-type plasminogeen activator (rt-PA) en welke veranderingen in endogeen urokinase-type plasminogeen activator (u-PA) kunnen optreden.

In deze open, gerandomiseerde studie kregen 8 gezonde niet rokende mannelijke vrijwilligers een continu infuus van 24 mg rt-PA over 160 minuten. Tegelijkertijd werd een infuus toegediend van 92 mg indocyanine groen (ICG) om indirect een maat te hebben voor de leverbloeddoorstroming. Op de ene studiedag werd 60 minuten na het starten van de infusen een gestandaardiseerde maaltijd genuttigd om de leverbloeddoorstroming te verhogen. Op de andere studiedag bleven de vrijwilligers gedurende de hele periode nuchter. Plasma spiegels van ICG, t-PA antigeen, t-PA activiteit, totaal u-PA antigeen, door plasmine mogelijk geactiveerd enkelketenig u-PA (scu-PA), actief tweeketenig u-PA (tcu-PA), fibrinogeen, afbraakprodukten van fibrine en fibrinogeen (TDP) en α_2-antiplasmine werden gemeten.

Na de consumptie van de maaltijd was de gemiddelde ICG concentratie 35%, t-PA antigeen concentratie 15% en t-PA activiteit 11% significant lager in vergelijking met de gemiddelde concentraties na het onthouden van voedsel. Geen veranderingen konden worden gemeten in fibrinogeen, TDP en α_2-antiplasmine concentraties na het innemen van voedsel. Het infunderen van rt-PA veroorzaakte een vijfvoudige stijging in actief tcu-PA concentraties en een tegelijkertijd optredende daling van meer dan 50% in scu-PA concentraties.

Uit deze studie kan worden geconcludeerd dat een verhoogde bloeddoorstroming van de lever resulteert in een toegenomen t-PA klaring,

alhoewel, zoals theoretisch al verwacht, de stijging in t-PA klaring proportioneel kleiner is dan de stijging in leverbloeddoorstroming. De omzetting van scu-PA in actief tcu-PA wordt verhoogd door het infunderen van rt-PA maar totaal u-PA antigeen concentraties blijven onveranderd.

Hoofdstuk 5

Het recombinant enkelketenig urokinase-type plasminogeen activator saruplase wordt voor een groot deel door de lever geklaard. Een grote interindividuele variatie in saruplase concentraties wordt er gezien bij patiënten met een acuut myocardinfarct. Variatie in hartfunctie na een infarct zou verschillen in leverbloeddoorstroming kunnen induceren, die de diversiteit in concentraties zou kunnen verklaren. Het onderzoek beschreven in dit hoofdstuk werd uitgevoerd om de relatie te onderzoeken tussen leverbloeddoorstroming en de farmacokinetische en farmacodynamische kenmerken van saruplase.

In deze open studie werden 13 patiënten opgenomen met een acuut myocardinfarct. Allen kregen ze een bolus injectie van 20 mg saruplase toegediend, gevolgd door een infuus van 60 mg saruplase over 1 uur. Voor het meten van de leverbloeddoorstroming werd tegelijkertijd 36 mg indocyanine groen (ICG) toegediend. Bloedmonsters werden afgenomen voor het meten van plasma spiegels van urokinase-type plasminogeen activator (u-PA) antigeen en activiteit, tweeketenig u-PA (tcu-PA) activiteit, ICG, fibrinogeen, afbraakprodukten van fibrine en fibrinogeen, α_2-antiplasmine en trombine anti-thrombine III complex.

Er werd een correlatie gevonden tussen ICG klaring en zowel u-PA antigeen als u-PA activiteit klaring. De tcu-PA activiteit was negatief gecorreleerd met fibrinogeen en α_2-antiplasmine concentraties.

De resultaten van deze studie laten zien dat de leverbloeddoorstroming in belangrijke mate de klaring van u-PA antigeen en activiteit bepaald en dat een verlaagde bloeddoorstroming in patiënten met hartfalen zal leiden tot een verhoging in plasma concentraties. De plasma concentraties van tcu-PA activiteit zijn geassocieerd met systemische fibrinogenolyse. Deze resultaten zouden gebruikt kunnen worden voor het optimaliseren van de saruplase behandeling in patiënten met een verminderde cardiale functie of om richtlijnen te ontwikkelen voor het gelijktijdig gebruik van andere geneesmiddelen die de leverbloeddoorstroming beïnvloeden.

Hoofdstuk 6

Het verwijderen van recombinant weefsel-type plasminogeen activator (rt-PA) uit het bloed door de lever is zo snel dat de lever de snelheidsbepalende stap is voor de klaring. In patiënten met een myocardinfarct kan de leverbloeddoorstroming

wisselen afhankelijk van de cardiale functie en de medicamenten die op dat moment toegediend worden. In deze studie werd in patiënten het effect van variatie in leverbloeddoorstroming op t-PA plasma concentraties gedurende trombolytische therapie bepaald.

Vijftien patiënten met een acuut myocardinfarct werden bestudeerd. Ze kregen de standaard trombolytische behandeling van rt-PA van 100 mg over 3 uur. Indocyanine groen (ICG) werd tegelijkertijd gegeven als maat voor de leverbloeddoorstroming, die ook door middel van Doppler echografie werd bepaald. Concentraties van t-PA antigeen, t-PA activiteit, ICG, α_2-antiplasmine, fibrinogeen en afbraakprodukten van fibrine en fibrinogeen werden gemeten. De hartfrequentie, bloeddruk en Killip classificatie werden gemeten als maat voor de klinische conditie van de patiënt.

ICG klaring en de klaring van zowel t-PA antigeen als t-PA activiteit waren positief aan elkaar gerelateerd. Er werden ook significante associaties gevonden tussen t-PA antigeen en afbraakprodukten van fibrine en fibrinogeen en tussen t-PA antigeen en α_2-antiplasmine.

Deze studie toont aan dat veranderingen in leverbloeddoorstroming in patiënten met een myocardinfarct omgekeerd gecorreleerd zijn met t-PA plasma concentraties. Hierdoor kan het voorkomen dat bij patiënten met ernstig verminderde hartfunctie zeer hoge t-PA plasma concentraties kunnen voorkomen als een standaard dosering wordt gegeven. Deze resultaten zouden kunnen leiden tot een optimalisering van de rt-PA dosering door het vaststellen van de klinische condities of interventies die de leverbloeddoorstroming beïnvloeden.

Hoofdstuk 7

In dit hoofdstuk wordt gesproken over de rol van C1-remmer als remmer van het weefsel-type plasminogeen activator (t-PA) in humaan plasma. Om de complexen van t-PA en C1-remmer te kunnen meten werd een 'enzym immuno assay' ontwikkeld. In vitro experimenten werden uitgevoerd met als resultaat dat gezuiverd C1-remmer met zowel enkel als dubbelketenig t-PA reageert. In plasma werd tweeketenig t-PA nauwelijks geremd door C1-remmer, terwijl enkelketenig t-PA in een zelfde mate werd geremd door endogeen C1-remmer als door gezuiverd C1-remmer. In vivo kon maar in enkele gevallen t-PA/C1-remmer complex gemeten worden in gezonde vrijwilligers, na een inspanningsproef en na een infuus met desmopressine. Het complex werd echter altijd in plasma gevonden na veneuze occlusie, in peritoneaal vloeistof van patiënten met 'peritoneal inflammatory disease' en in plasma van gezonde vrijwilligers gedurende een t-PA infuus. In het laatste geval werd bij de maximale t-PA concentratie ongeveer 8% van de geïnfundeerde hoeveelheid rt-PA geremd door C1-remmer. De halfwaardetijd van t-PA antigeen in plasma werd niet veranderd door de binding aan C1-remmer.

Uit deze studie kan dan ook geconcludeerd worden dat het in vivo

t-PA/C1-remmer complex meestal gevonden kan worden als het t-PA ontsnapt aan de snelle klaring van de lever en zich ophoopt in een plaats of als het in hoge concentraties in het bloed voorkomt.

Hoofdstuk 8

In dit hoofdstuk wordt de in vitro en in vivo complexvorming tussen t-PA en zijn langzame remmers beschreven. De vraag was of verschillen in concentraties van gebonden t-PA een verklaring zou kunnen zijn voor de variabiliteit in bloed concentraties van t-PA in patiënten die een behandeling kregen met een trombolytisch geneesmiddel. Remming van t-PA door gezuiverd α_2-antiplasmine werd onderzocht met een nieuw ontwikkelde bepaling, een zogenaamde 'sandwich immuno assay'. In plasma bleek 31% van het toegevoegde t-PA gebonden te zijn aan α_2-antiplasmine na 8 uur incubatie bij 37°C.

Complexvorming werd ook bestudeerd door het vrije van het gebonden t-PA te scheiden door middel van gelfiltratie. Zonder pre-incubatie bij 37°C was 87% van het toegevoegde t-PA vrij, terwijl na 6 uur het meeste t-PA (70%) was geremd, voornamelijk door C1-remmer en α_2-antiplasmine. Complexvorming van t-PA met C1-remmer en α_2-antiplasmine in patiënten met een trombolytische behandeling werd geanalyseerd in een bloedmonster dat 36 minuten na de start van het rt-PA infuus werd afgenomen. Theoretisch was berekend dat ongeveer 8.5% van de geïnfundeerde dosis in complex zou zijn met beide remmers. Door de snelle klaring van de complexen was echter maar 2% gebonden aan C1-remmer of α_2-antiplasmine. Onverwacht werd er na gelfiltratie nog een deel van het circulerende t-PA (8%) gevonden met een hoger molecuulgewicht dat nog steeds actief was.

Uit deze studie blijkt dat complexvorming van t-PA niet een belangrijke factor is bij de geobserveerde variabiliteit in bloedconcentraties van t-PA.

Hoofdstuk 9

Observaties uit een andere studie suggereerden een afwijkende farmacokinetiek van recombinant t-PA in de vroege fase van een infuus met een zeer lage dosis rt-PA. Vanwege het concave profiel van de plasma-concentratie curve vlak na het begin van de toediening van rt-PA leek het of een gedeelte van het geïnfundeerde rt-PA de circulatie niet bereikte en zich misschien gebonden had.

Daarom werd in een open gerandomiseerde studie twee lage dosis infusen van rt-PA toegediend aan 10 gezonde vrijwilligers. Elke proefpersoon kreeg éénmaal een dosis van 150 μg rt-PA in 40 minuten en éénmaal een dosis van 300 μg over dezelfde tijdsperiode. Er werden bloedmonsters afgenomen voor het meten van t-PA antigeen concentraties. Pre- en post-infusie data werden gebruikt om een voorspelling te kunnen doen van het concentratieprofiel gedurende de infusie

zonder dat er binding van t-PA zou optreden en deze waarden werden vergeleken met de werkelijk gevonden metingen met de t-PA binding.

Na het toedienen van 150 μg rt-PA werd er over de periode van het infunderen een significant verschil gevonden tussen de voorspelde en waargenomen gemiddelde t-PA concentraties. Na 300 μg rt-PA was het verschil tussen de voorspelde en waargenomen waarden niet meer significant. De gemiddelde hoeveelheid rt-PA dat werd gemist was 28 μg na het infuus van 150 μg en 21 μg na het infuus van 300 μg.

Deze resultaten duiden aan dat t-PA in een tot nu toe nog onbekend compartiment wordt gebonden. Mogelijkerwijs bindt het t-PA zich aan het endotheel. De techniek met de lage dosis infusen is mogelijk van waarde bij het verklaren van deze verdeling in verschillende patiëntengroepen en zou relevant kunnen zijn in relatie tot het meten van niet functionerend endotheel.

Hoofdstuk 10

In dit hoofdstuk worden de verschillende conclusies van iedere studie nogmaals besproken. Tevens wordt nagegaan wat voor onderzoek moeten worden verricht om de gedurende dit onderzoek bijgekomen vraagstellingen optimaal te kunnen beantwoorden.

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CURRICULUM VITAE

Jean van Griensven werd geboren op 26 juli 1963 te Rijswijk. Het V.W.O. diploma werd in 1981 behaald aan de Rijswijkse Openbare Scholengemeenschap. In datzelfde jaar werd een aanvang gemaakt met de studie Biologie aan de Rijksuniversiteit te Leiden. Na uiteindelijk te zijn ingeloot begon hij in 1982 met de studie Geneeskunde aan de Erasmus Universiteit te Rotterdam. Het propaedeutisch examen werd afgelegd in 1983, het doctoraal examen in 1987 en het artsexamen in 1989. Van augustus 1985 tot juli 1987 was hij werkzaam als studentassistent op de afdeling Experimentele Radiodiagnostiek aan de Erasmus Universiteit te Rotterdam. Van april 1985 tot augustus 1987 was hij eveneens werkzaam als lid van het medisch studententeam in het Sophia Kinderziekenhuis te Rotterdam. Sedert november 1989 is hij werkzaam als arts-onderzoeker bij het Centrum voor Humaan Geneesmiddelonderzoek te Leiden, waar in mei 1991 werd aangevangen met het in dit proefschrift beschreven onderzoek dat hier onder leiding van Prof. Dr. A.F. Cohen werd uitgevoerd. Van november 1991 tot september 1994 was hij werkzaam als avonddocent anatomie/fysiologie aan de Opleidingsschool voor Ziekenverzorgenden Zorghage te 's-Gravenhage. Sinds juni 1992 is hij als doseringsarts werkzaam bij de Trombosedienst te Leiden.