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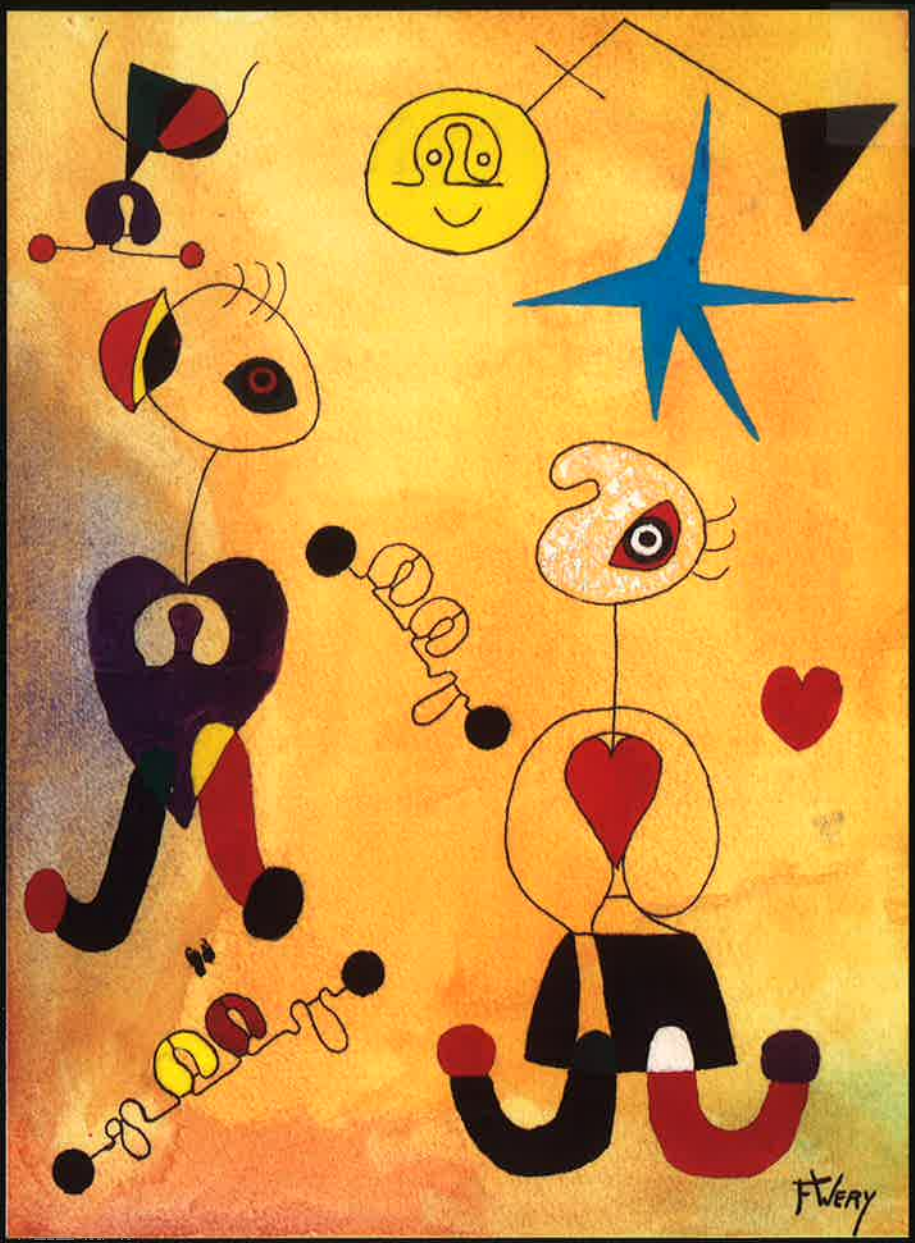
MOLECULAR WEIGHT SINGLE-CHAIN UROKINASE-TYPE PLASMINOGEN ACTIVATOR



FIBRINOLYTIC PROPERTIES OF LOW AND HIGH

Gé de Munk

Fibrinolytic properties of low and high molecular weight single-chain urokinase-type plasminogen activator

CIP-GEGEVENS KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Munk, Gerardus Adrianus Willem de

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Stellingen

1. Op biochemische gronden moet laagmoleculairgewicht scu-PA beschouwd worden als een efficiënt fibrinolytisch enzym, dat op zijn minst even geschikt kan zijn als hoogmoleculairgewicht scu-PA voor trombolytische therapie.
(dit proefschrift)

2. Het trombine-trombomoduline complex is een krachtige inactivator van scu-PA.
(dit proefschrift)

3. Kuo & Bjornsson gaan met hun "solid-phase immunoassay" geheel voorbij aan het feit dat u-PA in plasma voornamelijk in de één-ketenige vorm voorkomt.
Kuo & Bjornsson (1993) Anal. Biochem. 209, 70-78

4. In hun studie om aan te tonen dat de stimulatie door het fibrinogeen fragment FCB-2 van de activering van plasminogeen door scu-PA niet samenhangt met de activering van scu-PA, laten Kirchheimer *et al.* zien dat gevormd ^{125}I-tcu-PA inactief is, hetgeen het gehele onderzoek hoogst onbetrouwbaar maakt.
Kirchheimer et al. (1987) Eur. J. Biochem. 166, 393-397

5. Het door Sumi voorgestelde gebruik van lovastatine (een inhibitor van de cholesterol synthese) als cytostaticum is niet aan te bevelen.
Sumi et al. (1992) Gastroenterology 103, 1717-1724

6. Bij het ontwikkelen van behandelingsstrategieën van een aantal autoimmuunziekten met antibodies gericht tegen het CD4 molecuul wordt onvoldoende rekening gehouden met het feit dat dit molecuul ook op de hematopoietische stamcel tot expressie komt.
Wraith et al. (1989) Cell 57, 709-715
Wineman et al. (1992) Blood 80, 1717-1724

7. Het feit dat de symbiontische bodembacteriën van de geslachten *Rhizobium, Bradyrhizobium* en *Azorhizobium* gebruik maken van chitine-achtige lipo-oligosaccharides voor signaal overdracht naar de nog ongeïnfecteerde gastheerplant, doet vermoeden dat deze stoffen een algemene rol spelen als groeifactor in de morphogenese van hogere planten.
Spaink et al. (1993) In: *Advances in Molecular Genetics of Plant-Microbe Interactions. Nester and Verma (eds). Kluwer Academic Publishers, The Netherlands. 151-162*

8. Romans van vrouwelijke auteurs zijn in wezen ongeschikt voor de tot nu toe gangbare structuuranalysemodellen, die erop gericht zijn het mysterie van het verhaal te ontrafelen.

9. Bezuinigingen door het Ministerie van Justitie op de eerstelijnsrechtshulp bij de buro's voor rechtshulp door middel van invoering van een bijdrage van f 30,- na het eerste halve uur gratis hulpverlening, zal juist een kostenverhogend effect hebben op de post rechtshulp, doordat het aantal gerechtelijke procedures zal toenemen.

10. Dankzij het succes van de biologische bestrijding van schadelijk ongedierte in de tuinbouw, nu in 90 % van de kassen toegepast, kan het hoofd worden geboden aan de toegenomen plaagdruk en de hogere productie eisen zonder toename van het gebruik van chemische bestrijdingsmiddelen.

11. Het toenemend toerisme van europeanen naar ontwikkelingslanden rechtvaardigt een soepeler beleid bij de afgifte van reisvisa voor landen in Europa aan onderdanen van deze ontwikkelingslanden.

12. Het raadsel van de kip en het ei (Andreotti, 1992) is achterhaald sinds de publicatie van "The origin of species by means of natural selection" door C. Darwin (1859), waarin hij concludeert dat elke diersoort niet onafhankelijk is geschapen, maar afstamt, als een variatie, van andere soorten. Eierbarende dieren, en dus eieren, bestonden al voordat vogels, en dus ook kippen, op de wereld verschenen.
Andreotti (1992) Proefschrift, statement 15. Rijksuniversteit Leiden
Darwin (1859) The origin of species by means of natural selection. J.Murray, London

13. Op het wereldtoneel is militair ingrijpen niet geschikt voor de rol van *deus ex machina*.

14. Een brakman koestere zijn brakken.

Stellingen bij het proefschrift: Fibrinolytic properties of low and high molecular weight single-chain urokinase-type plasminogen activator.

Gé A.W. de Munk, Leiden, 29 juni 1993

Fibrinolytic properties of low and high molecular weight single-chain urokinase-type plasminogen activator

proefschrift

ter verkrijging van de graad van Doctor
aan de Rijksuniversiteit te Leiden,
op gezag van de Rector Magnificus Dr. L. Leertouwer,
hoogleraar in de faculteit der Godgeleerdheid,
volgens besluit van het College van Dekanen
te verdedigen op dinsdag 29 juni 1993
te klokke 15.15 uur

door

Gerardus Adrianus Willem de Munk

geboren te Lisse in 1960

Promotiecommissie:

Promotor:	Prof. dr. P. Brakman
Co-promotor:	Dr. D.C. Rijken (IVVO-TNO, Gaubius Laboratorium)
Referent:	Prof. dr. H.R. Lijnen (Katholieke Universiteit Leuven)
Overige leden:	Prof. dr. Th.J.C. van Berkel
	Prof. dr. R.M. Bertina
	Prof. dr. B.N. Bouma (Rijksuniversiteit Utrecht)

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Financial support by the Netherlands Heart Foundation and IVVO-TNO, Gaubius Laboratory, Leiden, for the publication of this thesis is gratefully acknowledged.

Cover: Miró's Enzymes, Fleur Wery

Tot het duistere en onbekende gaan,
door wat nog duisterder en onbekender is.

Alchemistisch devies

Aan mijn ouders

CONTENTS

CHAPTER 1

INTRODUCTION

1.1 The role of fibrinolysis in the haemostatic balance

Following vascular damage, three processes prevent bleeding: 1) leakage of blood is restricted by the narrowing of vessels, a process which is called vasoconstriction, 2) a haemostatic plug is formed by platelets which bind to the damaged vessel wall and aggregate, and 3) an insoluble network of fibrin is formed by a fast and effective biochemical process, known as coagulation. These three processes interact with each other. Activated platelets release factors which stimulate coagulation (for example: factor Va, platelet factor 4 and fibrinogen) and vasoconstriction (serotonine and adrenalin), bind fibrin(ogen) via specific receptors and change their phospholipid surface into a pro-coagulant one. Coagulation activates platelets (Mann et al. 1987) and promotes vasoconstriction (Busse and Pohl 1988) by the action of thrombin.

Fibrinolysis is the antagonistic biochemical process of coagulation. Fibrinolysis degrades the insoluble fibrin network into soluble fibrin degradation products. Both processes, fibrinolysis and coagulation, together determine the extent and life span of a thrombus. In healthy subjects both processes are in a steady state with low levels of active components, as reflected by the presence of proteolytic products of coagulation and fibrinolysis in plasma. Indicators for ongoing coagulation are: 1) the prothrombin fragment F1+2, which is found after activation of prothrombin (Aronson et al. 1977), 2) thrombin-antithrombin III complexes (Teitel et al. 1982) and 3) fibrinopeptide A, which is split off from fibrinogen by thrombin (Nossel et al. 1976). Indicators for fibrinolysis are 1) the plasmin-α_2-antiplasmin complex (Holvoet et al. 1986) and 2) peptide Bβ15-42 which is released from fibrin by plasmin (Nossel 1981) or other fibrin degradation products (Nieuwenhuizen 1991). Fibrinolysis is in a delicate balance with coagulation. When this haemostatic balance is disturbed by the impairment of coagulation or by the stimulation of fibrinolysis, a bleeding tendency may be found. Impairment of fibrinolysis or stimulation of coagulation may lead to fibrin deposition, resulting in thrombosis (Astrup 1958).

1.2 Other roles of the plasminogen activation system

The enzymes of the fibrinolytic system are also involved in other physiological and pathological processes. It has been shown that plasminogen activators are involved in ovulation (Cajander et al. 1989), embryogenesis (Sappino et al. 1989), neural development (Seeds et al. 1991), wound healing (Clowes et al. 1990), angiogenesis (Gross et al. 1983), inflammation and rheumatoid arthritis (Werb et al. 1977) and destructive cancer growth and tumour metastasis (Danø et al. 1985). Based on the widespread occurrence of fibrinolytic components, it can be supposed that they also have functions in other processes but these often remain unclear. Plasminogen activation may result in fibrin degradation or activation of pro-enzymes of metalloproteases, which can degrade the extracellular matrix. Cleavage of fibronectin directly by urokinase-type plasminogen activator has been described (Gold et al. 1989). Binding of plasminogen and plasminogen activators to specific receptors can localize the proteolytic activity to specific micro-environments or cell surfaces (Miles et al. 1988).

1.3 The biochemical mechanism of coagulation

Fibrinogen is the final substrate in blood coagulation (Fig. 1). Fibrinogen is a large glycoprotein with a Mr of 340,000 and consists of two Aα-, two Bβ- and two γ-chains linked together by disulphide bonds. Thrombin cleaves the fibrinopeptides A and B from fibrinogen, resulting in the formation of fibrin monomers. The fibrin monomers polymerize to an insoluble fibrin network (reviewed by Doolittle 1981). The precursor of thrombin, prothrombin, is activated by the prothrombinase complex formed by factor Xa, factor Va, phospholipids and Ca^{2+}. The required phospholipid surface comes from damaged vascular cells, the inside of broken red blood cells or activated platelets. Factor X is activated by proteases of the extrinsic pathway (factor VIIa/tissue factor) or the intrinsic pathway (factor IXa/VIIIa). The extrinsic pathway is triggered by the tissue factor (thromboplastin) present on the subendothelium, smooth muscle cells and fibroblasts. The intrinsic pathway is initiated by thrombin, as a feedback mechanism (activation of factor XI) or by plasma proteins (factor XII and prekallikrein) activated by a foreign surface (see Fig. 1). The two pathways are not strictly divided. Factor IXa of the intrinsic pathway can activate factor VII of the extrinsic pathway, and the extrinsic factor VIIa can activate factor IX of the intrinsic pathway (Mann et al. 1988, Davie et al. 1991).

At least five physiological inhibitors of blood coagulation are known: 1) the heparin-dependent Antithrombin III, which inhibits thrombin and factors IXa, Xa and XIa, 2) the

heparin-dependent Heparin Cofactor II, which inhibits thrombin, 3) Tissue Factor Pathway Inhibitor (TFPI also called LACI or EPI) which inhibits factor Xa, and subsequently can inhibit the factor VIIa/tissue factor complex, 4) C_1-inhibitor, which inhibits factor XIIa and kallikrein and 5) Thrombomodulin, a membrane-bound protein which binds thrombin and inhibits thrombin in the formation of fibrin and the activation of platelets, factor V and factor VIII. Moreover, thrombomodulin acts as a cofactor in the inhibition of thrombin by Antithrombin III. Antithrombin III and Heparin Cofactor II are members of the Serpin (Serine Protease Inhibitor) family of proteins. TFPI is a multivalent Kunitz-type inhibitor.

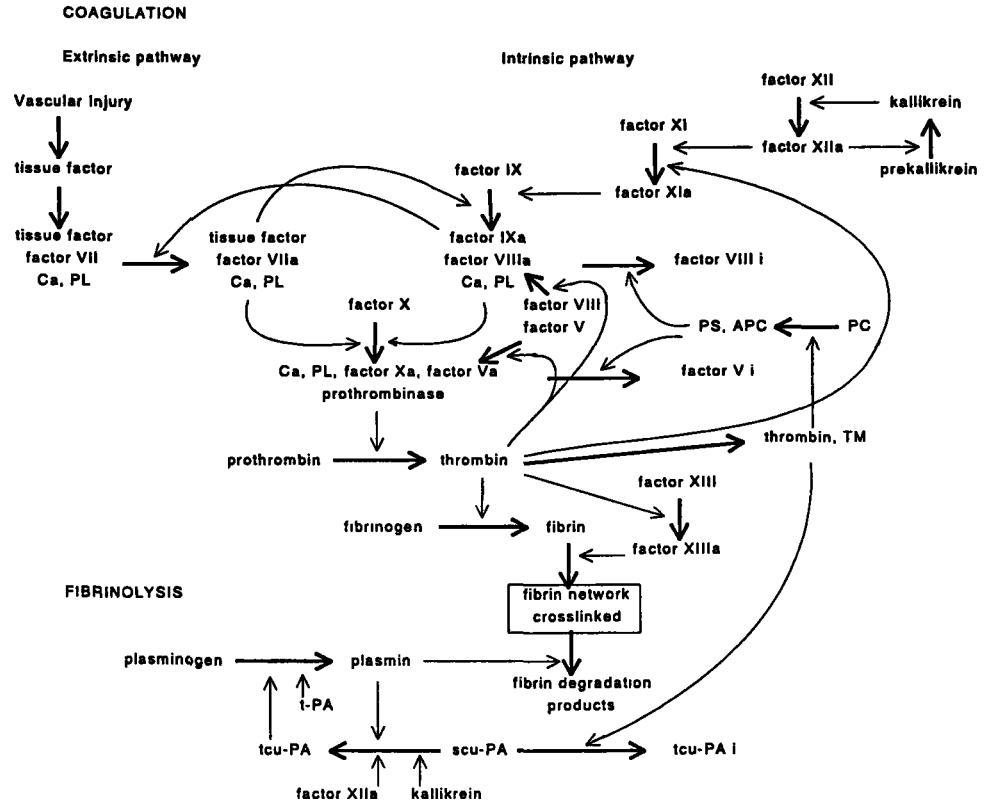

Figure 1. Coagulation cascade and the fibrinolytic system. Formation and degradation of an insoluble crosslinked fibrin network by respectively coagulation and fibrinolysis. The bold arrows indicate conversion, the thin arrows indicate enzymatic activity. Activated clotting factors are designated by lower case a, inactivated factors or enzymes are designated by a lower case i. Abbreviations: PL, phospholipids; Ca, Ca^{2+} ions; u-PA, urokinase-type plasminogen activator; t-PA, tissue-type plasminogen activator; sc, single-chain; tc, two-chain; PC, protein C; PS, protein S, APC, activated protein C; TM, thrombomodulin.

Coagulation is also inhibited by a negative feedback mechanism. Thrombin bound to thrombomodulin activates protein C. Activated protein C inactivates factor Va and factor VIIIa, thereby reducing the generation of thrombin (Esmon 1989). Protein S is thought to be a cofactor of activated protein C, although it recently has been shown to have also independent anticoagulant activity (Heeb et al. 1993). Activated protein C can be inhibited by Protein C Inhibitor/Plasminogen Activator Inhibitor-3, α_2-antitrypsin and α_2-macro-globulin.

The function of the so-called clotting cascade in which different proteases activate each other is: 1) to produce a large amount of thrombin in a short time upon a small trigger, 2) to make close tuning possible regarding the exact need of thrombin by several feedback, stimulation and inhibition steps.

1.4 The biochemical mechanism of fibrinolysis

The fibrinolytic system is based on the same cascade principle as the coagulation system. The insoluble fibrin network is degraded into soluble fibrin degradation products by the active protease plasmin. Plasmin is formed by specific hydrolysis of the Arg561-Val562 bond of plasminogen by a plasminogen activator (PA). Plasminogen has a Mr of 92,000 and its concentration in plasma is 2 μM (Table 1). Two PAs in human plasma are known: urokinase-type PA (u-PA) and tissue-type PA (t-PA). There is some evidence for the presence of a third type, factor XII-dependent PA (Kluft et al. 1979, Binnema et al. 1990). As early as a hundred years ago (Sahli 1885), and more recently 50 years ago (MacFarlane et al. 1947), u-PA was found in urine, and was shown to be able to dissolve fibrin clots. Sobel at al. (1952) showed that this enzyme acts via the activation of plasminogen and introduced the name urokinase. Urine is still a main source of pharmaceutical u-PA preparations. The concentration of u-PA in urine is 1-10 nM, in plasma about 150 pM. Later on, t-PA was identified in tissue extracts (Astrup & Stage 1952) and characterized from porcine heart tissue (Wallén 1977) and human uterine tissue (Rijken et al. 1979). t-PA has a Mr of about 70,000, its plasma concentration under basal conditions is about 70 pM. Both t-PA and u-PA are released in plasma in a single-chain form. In contrast to other serine proteases, t-PA is equally active in its single- and two-chain form. Single-chain u-PA (scu-PA) or pro-urokinase has a very low activity towards plasminogen and small chromogenic substrates. Plasmin, factor XIIa, plasma kallikrein and probably some other enzymes can activate scu-PA by hydrolysing the Lys158-Ile159 bond.

The fibrinolytic system has a number of physiological inhibitors. Plasmin is inhibited by α_2-antiplasmin (plasma concentration: 1 μM) and α_2-macroglobulin. The PAs are

inhibited by Plasminogen Activator Inhibitors 1-3 (PAI-1, PAI-2, PAI-3 respectively) and Protease Nexin (Kruithof 1988). All four inhibitors are members of the Serpin family of proteins, and have an Arg at the P1 position of the reactive site. PAI-1 is the primary inhibitor of both t-PA and u-PA in normal plasma. Its plasma concentration of about 1 nM increases considerably by release from platelets during aggregation of the platelets. PAI-1 is also found in extracellular matrices of different cell types. PAI-2 inhibits two-chain t-PA as well as u-PA, but is a slow inhibitor of active one-chain t-PA. PAI-2 concentration in normal plasma is very low or undetectable. PAI-2 has been detected in plasma during pregnancy, in leucocytes and placental tissue. Nonglycosylated PAI-2 is found in large quantities intracellularly, where its role is unknown. PAI-3 is identical to Protein C Inhibitor, and inhibits u-PA, activated protein C, thrombin and, at a slow rate, t-PA. The plasma concentration of PAI-3 is relatively high: 100 nM. Protease Nexin is a broad spectrum inhibitor. It inhibits t-PA and u-PA and is also a fast inhibitor of plasmin and thrombin.

Table 1. Physicochemical properties of components of the fibrinolytic system.

	MW[1] kD	Chain(s)	plasma concentration mg/l	M	carbohydrate percent
plasminogen	92	1	200	2 μM	2
u-PA	54	1	0.008	150 pM	7
t-PA	70	1	0.005	70 pM	7/13
prekallikrein	88	1	40	450 nM	15
factor XII	80	1	30	375 nM	17
α_2-antiplasmin	70	1	70	1 μM	13
α_2-macroglobulin	725	4	2,500	3 μM	8
PAI-1	52	1	0.05	1 nM	
PAI-2	46/70	1	< 0.005	< 100 pM	
PAI-3	57	1	5	100 nM	

[1] As determined by SDS-PAGE. Abbreviations u-PA, urokinase-type plasminogen activator; t-PA, tissue-type plasminogen activator; PAI, plasminogen activator inhibitor. (Modified from Bachmann (1987)).

The presence of fibrin is essential to the activation of plasminogen (Lijnen & Collen 1982). Fibrin initiates, stimulates and prevents the inhibition of the fibrinolytic activity. Fibrin acts as follows: 1) it binds via its internal and, after limited digestion, via its COOH-terminal lysine residues t-PA and plasminogen, resulting in a stimulation of the

activity of t-PA, 2) it changes the conformation of plasminogen in such a way that it can be activated more quickly by scu-PA, 3) it protects bound plasmin from inhibition by α_2-antiplasmin. As a result fibrin will be degraded selectively, without damage to other plasma proteins.

As well as fibrinolysis-promoting fibrin, a thrombus also contains inhibitors of the fibrinolytic system. These include α_2-antiplasmin and PAI-1, which bind to the fibrin network. Platelet-rich thrombi are more resistant to thrombolysis than platelet-poor thrombi. An important mechanism of the inhibition of thrombolysis by platelets is the thrombin-dependent release of PAI-1. The fibrin-bound inhibitors must first be neutralized by active fibrinolytic enzymes before fibrinolysis can start. Thrombin involved in the formation of the thrombus remains bound to the fibrin-network, and may inactivate scu-PA.

Protein C, activated by thrombin in complex with cofactor thrombomodulin, stimulates fibrinolysis in a way which has yet to be elucidated (Zolton et al. 1973). It has been proposed that activated protein C neutralises PAI-1 (Van Hinsbergh et al. 1985), but this reaction appears to be too slow to be of major physiological significance (Kruithof 1988). Moreover, protein S does not enhance this reaction while protein S as a cofactor for activated protein C is necessary for the profibrinolytic effect of activated protein C in a whole blood clot lysis assay in vitro (De Fouw et al. 1986). The profibrinolytic effect of activated protein C is probably dependent on the inhibition of the generation of thrombin by activated protein C (de Fouw 1988, Bertina et al. 1992).

1.5 The molecular architecture of coagulation and fibrinolytic proteases

All proteolytic enzymes of coagulation and fibrinolysis are serine proteases, none of the enzymes belongs to any of the three other classes of proteases: the thiol proteases, the metalloproteases or the carboxyl (or acid) proteases. The serine proteases also include the digestive enzymes trypsin and chymotrypsin. In the active centre, three amino acid residues, together forming the "catalytic triad": Ser, His and Asp play an essential role. In the inactive single-chain form of a serine protease the primary binding pocket is closed and the catalytic triad is in an inactive conformation. Cleavage of the protein at a single specific site leads to a new NH_2-terminus with two aliphatic hydrophobic amino acids followed by two Gly residues. The non-catalytic chain remains fixed to the catalytic chain by one or more disulphide bridges. The new NH_2-terminus intrudes the enzyme and forms an ion pair between the free α-amino group and the acidic side chain of the Asp adjacent to the Ser of the active centre. A conformational change opens the primary

substrate binding pocket and brings the catalytic triad into the proper arrangement (Blow et al. 1969).

In order to bind to other macromolecules, the proteases of coagulation and fibrinolysis have non-catalytic domains attached to the NH_2-terminal end of the trypsin-homologues protease domain (Furie & Furie 1988). These non-catalytical domains are autonomous in structure, function and folding. Four main types are distinguishable: 1) the vitamin K-dependent Ca^{2+} binding domain with γ-carboxylated Glu residues, 2) the *kringle domain*, 3) the epidermal growth factor domain and 4) the *finger domain*. The three fibrinolytic enzymes (Fig. 2) are built up as follows: at the NH_2-terminal, t-PA has a propeptide followed by a fibrin-binding finger domain, an epidermal growth factor domain, a kringle of unknown function and a second kringle which binds both internal and COOH-terminal lysine residues of fibrin. The COOH-terminus of t-PA consists of the proteolytic domain. Between the propeptide and the protease domain, u-PA has an epidermal growth factor domain which binds to the u-PA specific receptor, and a kringle domain of unclear function. Plasminogen has five kringles. The first and fourth kringle have a high-affinity COOH-terminal lysine binding site, the fifth kringle may bind to internal lysine residues of fibrin (Christensen 1984). The kringle domains also bind α_2-antiplasmin.

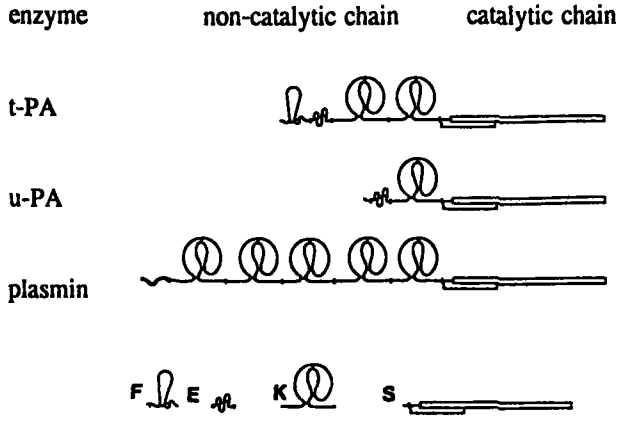

Figure 2. Building domains of fibrinolytic serine proteases. F: finger domain; E: epidermal growth factor domain; K: kringle domain; S: serine protease domain.

The coagulation and fibrinolytic enzymes are assumed to originate from an ancestral trypsin-like protease. During evolution, the non-catalytic domains were added between the signal peptide and the protease domain, probably by insertion of exons from genes of other proteins. The domains were internally-duplicated in some plasma proteases or

exchanged to other proteases, and evolved independently. Evolutionary dendrograms of the individual domains and the whole proteases have been constructed based on sequence homology (Patthy 1985).

1.6 Fibrinolytic therapy in acute myocardial infarction

An example of a local pathological disturbance of the haemostatic balance is the acute myocardial infarction (De Wood et al. 1980). An atherosclerotic plaque in the vessel wall narrows a coronary artery. The plaque consists of a core of extracellular lipid and necrotic debris covered by collagen and lipid-filled macrophages (Bierman 1991). A rupture in the cap of an atherosclerotic plaque allows contact between blood and the collagen. As a result, a thrombus can be formed suddenly (Richardson et al. 1989). The thrombus cuts off the oxygen and energy supply leading to death of part of the heart muscle. Acute myocardial infarction is the main cause of death in the western world. Quick recanalization within the first hours improves the patient's chances of survival. Recanalization can be achieved by administering plasminogen activators which lyse the thrombus (Rentrop et al. 1979).

Fibrinolytic therapy not always leads to the desired effect. In a quarter to a third of cases the therapy fails. Moreover, there is a risk of bleeding and of reocclusion after successful recanalization. Different thrombolytic drugs are in use: streptokinase (GISSI 1986, 1987), APSAC (anisoylated complex of streptokinase and plasminogen)(AIMS trial 1988), two-chain u-PA (Bell 1988) and t-PA (Wilcox et al. 1988, GISSI-2 1990, ISIS-3 1992). Other agents are still in the development stage: scu-PA, bat-PA from saliva of the vampire bat, staphylokinase, mutants of t-PA or scu-PA, hybrid molecules with domains of different fibrinolytic proteases and complexes of plasminogen activators with antibodies to fibrin or platelets (Collen & Lijnen 1991, Lijnen & Collen 1991).

1.7 Aim of the study

As a thrombolytic drug, scu-PA combines a number of favourable characteristics: it is a human protein and is therefore much less antigenic than bacterial proteins, it does not react with PAIs because of its zymogenic form, it is rather specific in fibrin degradation without degradation of fibrinogen or other proteins, and last but not least it is a fast acting and effective drug (PRIMI trial 1989, Loscalzo 1989). Low Molecular Weight (LMW) scu-PA is proteolytic derived by the metalloprotease Pump-1 from the normal

High Molecular Weight (HMW) scu-PA (Marcotte et al. 1992). Almost the entire non-catalytic A-chain has been cut off by proteolysis of the Glu143 - Leu144 bond, reducing the Mr from 54,000 to 33,000. LMW scu-PA lacks the epidermal growth factor domain which is responsible for binding to the specific u-PA receptor, as well as the kringle domain to which no clear function can yet be ascribed. It is assumed that the deleted domains have no primary function in fibrinolysis. LMW scu-PA may have similar thrombolytic properties as HMW scu-PA.

The aim of this study is 1) to investigate if LMW scu-PA is a potent fibrinolytic enzyme, and 2) to compare the properties of LMW and HMW scu-PA in reactions which are important for regulation of their fibrinolytic activity.

The second chapter presents a review of the fibrinolytic properties of scu-PA which were known at the beginning of the study. The third chapter describes the occurrence of different forms of u-PA in urine. Urine was considered a potential source for isolation of LMW scu-PA because u-PA concentrations are high and because both LMW u-PA and u-PA in zymogen form are present. Moreover LMW scu-PA has been found after purification of u-PA from conditioned kidney cell culture medium (Wijngaards et al. 1986), suggesting that, in principle, kidney cells can release LMW scu-PA in urine. During our studies it appeared that under specific conditions u-PA is inhibited by PAI-3/protein C inhibitor in urine. This reaction is strongly stimulated by urinary glycosaminoglycans. Chapter four describes the interaction between HMW and LMW u-PA and the glycosaminoglycan heparin, as well as the effect of heparin on the activation of plasminogen. Inactivation of scu-PA by thrombin can be an important reaction in the haemostatic balance. In chapter five, the effect of thrombomodulin on the inactivation of HMW and LMW scu-PA by thrombin is investigated. The role of the glycosaminoglycan side chain of thrombomodulin in this reaction is investigated in chapter six. In chapter seven the fibrinolytic activities of HMW and LMW scu-PA in different fibrinolytic assays are compared. During thrombolytic therapy large amounts of scu-PA are needed because of rapid clearance from the circulation by the liver. For that reason the clearance mechanisms of LMW and HMW scu-PA are compared in chapter eight.

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CHAPTER 2

FIBRINOLYTIC PROPERTIES OF SINGLE-CHAIN UROKINASE-TYPE PLASMINOGEN ACTIVATOR (PRO-UROKINASE)

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2.1 Introduction

Single-chain urokinase-type plasminogen activator (scu-PA) or pro-urokinase is the precursor of two-chain urokinase-type plasminogen activator (tcu-PA), a well-known enzyme which efficiently catalyses the conversion of plasminogen to plasmin.

Urokinase-type plasminogen activator (u-PA) is found in several body fluids and tissues and may have more than one physiological function. It probably plays an important role in cell migration, tissue destruction and remodelling (for reviews see 1-3). On many cell types, u-PA is bound to a specific plasma membrane receptor via the epidermal growth factor domain of the ligand (4-6). This u-PA receptor localizes the proteolytic activity of u-PA to the cell surface (review see 7). Although u-PA does occur in plasma (8-10), it is not yet known whether the u-PA system is involved in endogenous fibrinolysis. However, tcu-PA and recently also scu-PA, are successfully applied for fibrinolytic therapy. This review will summarize the fibrinolytic properties of scu-PA and will focus on molecular mechanisms underlying the thrombolytic action. Other reviews on scu-PA have been published elsewhere (11-16).

2.1 Production and purification

For a long time only tcu-PA was known. A latent u-PA activity was first detected in a kidney cell culture by Bernik (17) and Nolan et al. (18). This activity was activatable by trypsin or plasmin and was considered to be derived from a proactivator. Later on, scu-PA was purified from freshly voided urine (19-21), kidney cell cultures (22-25), various cancer cell cultures (26-32), human fibroblast cultures (33) and plasma (9,34). Recombinant scu-PA was produced both in E.coli cells (in an unglycosylated form) (35-37) and in Chinese hamster ovary cells (38) or COS-1 cells (39).

Different specific affinity techniques are used for purification (Table 1). Fibrin celite columns are applied specifically for scu-PA, as tcu-PA reveals a lower affinity for fibrin celite (19). Zinc chelate agarose (21,31) binds both scu-PA and tcu-PA. By contrast, benzamidine and agmatine agarose (20-23,31,36) only bind tcu-PA, probably through the active site, and not scu-PA. Immuno-affinity chromatography (9,20,21,24-27,29,31-33) is used, both with monoclonal and polyclonal antibodies.

The yield of scu-PA is about 5-25 μg per liter of urine, as only 10-30% of the total amount of u-PA antigen present in urine consists of scu-PA (19-21). In bladder urine from mouse more than 50% of total u-PA exists as scu-PA (40). A yield of 65-100 μg is obtained per liter of conditioned medium of a human lung adenocarcinoma line CALU-3

(31) and in addition, 56 μg of a low molecular weight form of scu-PA (41). Yields of scu-PA from other cancer cell lines are similar (26,27-30).

Table 1. Binding of single-chain and two-chain u-PA to specific affinity columns.

Column material	scu-PA	tcu-PA	References
Fibrin celite	+	±	19
Zinc chelate agarose	+	+	21,31
Benzamidine agarose	-	+	18,21,22,36
Agmatine agarose	-	+	20
Antibody agarose[1)]	+	+	9,20,21,24-27,29,31-33

[1)] Antibodies specific for single- or two-chain u-PA may be produced (134), but are not yet commonly used.

A rough estimate of u-PA in plasma is 3-10 μg/l (9,42). Application of specific immunoassays showed mean u-PA levels in plasma of 1-10 μg/l (43-45). About 50 μg apparently homogeneous, but partially activated scu-PA can be isolated from 3 kg Cohn fraction IV-1 (9).

2.3 Protein structure

Scu-PA is a single-chain glycoprotein with a Mr of approximately 54,000. The amino acid sequence of u-PA has been determined by amino acid sequence analysis (46-49) as well as by cDNA analysis (35,50) (Fig. 1). The chain contains 411 amino acids with 24 cysteine residues and a carbohydrate side chain at Asn 302. Four different domains can be distinguished on the base of the primary structure: 1) the epidermal growth factor domain; 2) the kringle domain; 3) the connecting peptide domain (together called the light- or A-chain), and 4) the proteolytic domain (called the heavy or B-chain). These domains have significant homology with domains found in other proteins and are probably evolution-related (51). The growth factor domain (amino acids 5-45) is related to murine and human epidermal growth factor and has a binding site for the u-PA receptor (5). The kringle domain (amino acids 46-134) is homologous to the kringles of prothrombin, plasminogen and tissue-type plasminogen activator (t-PA). The function of the kringle in u-PA is still unknown. In plasminogen, kringles play a role in fibrin

binding. In t-PA the second kringle is involved in fibrin binding and in stimulation of its proteolytic activity by fibrin (52,53). The connecting peptide domain (amino acid residues 136-158) and the proteolytic domain are also present in most other higher animal serine proteases such as t-PA, plasmin, thrombin, chymotrypsin, trypsin and elastase. After activation by cleavage of the Lys_{158} - Ile_{159} bond, the connecting peptide and the proteolytic domain remain connected by a disulphide bridge (Fig. 1).

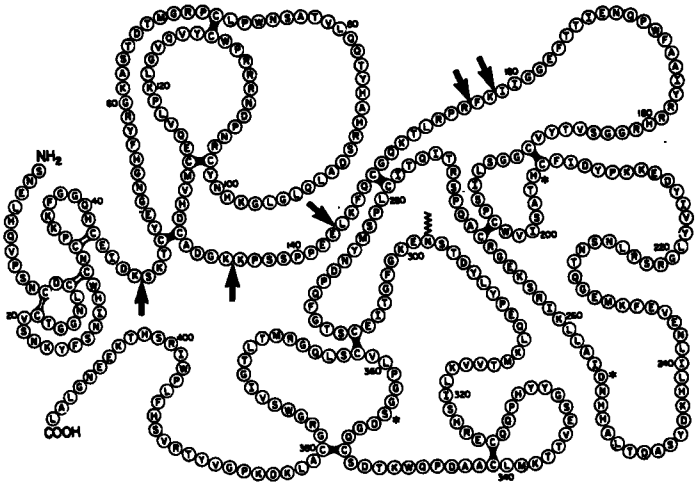

Figure 1. Two-dimensional structure of human single-chain urokinase-type plasminogen activator (scu-PA). The arrows indicate cleavage sites for various proteolytic enzymes (see text). Cleavage of the Lys_{158} - Ile_{159} bond results in the active two-chain form. The zig-zag line indicates the N-glycosylation site, and the stars show the three active site amino acid residues. (Modified from Holmes et al. (35).

The B-chain contains the amino acid residues His_{204}, Asp_{255} and Ser_{356} which probably form, homologous to the other serine proteases, the "catalytic triad" of the active centre. The primary substrate binding pocket, containing Asp_{350}, is probably identical to the pocket of trypsin (54) which is suitable for arginine and lysine-containing substrates. Activation of scu-PA to tcu-PA by cleaving the Lys_{158} - Ile_{159} bond leads to a new NH_2-terminal, which probably intrudes the proteolytic domain, as it does in other serine proteases, by forming an ion-pair between the α-amino group of Ile_{159} and the acidic side chain of Asp_{355} (55). In this way, a conformational change opens the substrate binding pocket and possibly the active site (56).

The C-terminal amino acid of the A-chain of urinary tcu-PA is Phe_{157} (47). As scu-PA has a Lys in position 158 (49) and cleaving by trypsin or plasmin always happens at the

C-terminal side of a Lys or Arg residue, another enzyme in urine should cut off Lys_{158} after the activation reaction has taken place.

The C-terminal amino acid of the A-chain is probably less important for the properties of tcu-PA. This has been shown by a $Lys_{158} \rightarrow Glu_{158}$ mutant of u-PA. Its tcu-PA form, obtained by cleavage with endoproteinase Glu-C (Staphylococcus aureus V8-protease) is fully active (57). Moreover, treatment of scu-PA with thrombin and a high concentration of plasmin successively results in a form which lacks Phe_{157} and Lys_{158} at the C-terminus of the A-chain. This form has the same properties as normal tcu-PA (58). On the other hand, removal of the C-terminal lysine in tcu-PA by carboxypeptidase B reduces the rate of plasminogen activation about 2-fold (59).

In addition to the activation cleavage site between Lys_{158} and Ile_{159}, u-PA has at least four more cleavage sites (see arrows in Fig. 1): 1) between Lys_{135} and Lys_{136} in tcu-PA, sensitive to plasmin (60) or another trypsin-like enzyme (61), and resulting in low molecular weight tcu-PA with Mr 33,000; 2) between Lys_{46} and Ser_{47}, resulting in a 40,000 species, probably observed during autocatalytic digestion of urinary tcu-PA (62) and abundantly present after plasmin treatment of scu-PA mutants with Lys_{135} replaced by Gln (63); 3) between Arg_{156} and Phe_{157} in scu-PA, sensitive to thrombin and resulting in an inactive tcu-PA (64); and 4) between Glu_{143} and Leu_{144} in scu-PA, resulting in a form of scu-PA with Mr 32,000 (41). It is still unknown whether these reactions occur *in vivo*. The inactivation of scu-PA by thrombin may delay thrombolysis during and immediately after clot formation (65,66) and thus reduce bleeding complications during thrombolytic therapy with scu-PA at sites with fresh clots and thrombin still present (12)*. On the other hand, inhibition of thrombin by the use of heparin may increase the thrombolytic efficacy of scu-PA (67,68).

2.4 Functional properties

Activation of scu-PA

Scu-PA can be activated by trypsin (17,18,64), plasmin (25,27,28,33,64,69) and plasma kallikrein (64,70). Other enzymes may also cleave the Lys_{158} - Ile_{159} bond, as suggested for instance by a study by Stephens et al. (71). These authors reported that leukemic cell lines produce predominantly tcu-PA, while cell lines derived from solid tumors produce mainly scu-PA. Leukemic cells apparently convert scu-PA to tcu-PA by producing an unknown enzyme with appropriate specificity (71). No activation of scu-PA is obtained by tissue

* Significant fibrinolytic activity *in vivo* of administered thrombin-cleaved scu-PA has been shown (135,136).

kallikrein and several other serine proteases of the coagulation system; only with factor XII is some activation accomplished (64). Which enzyme activates scu-PA *in vivo* is still unknown. Both kallikrein and plasmin are good candidates; they may produce tcu-PA during contact activation and plasminogen activation (positive feed-back mechanism), respectively.

Reaction with synthetic substrates and inhibitors

Scu-PA has no, or merely a low, amidolytic activity against small synthetic substrates. The amidolytic activity is at most 0.4% of the activity of tcu-PA (72).

Scu-PA, in contrast to tcu-PA, is resistant to irreversible inactivation by diisopropylfluorophosphate (DFP) (9,24,27-30,72) and by Glu-Gly-Arg-CH_2Cl (73). The latter compound acts as a reversible (competitive-type) inhibitor of the plasminogen activating activity of scu-PA (73). Scu-PA does not form SDS-stable complexes with PAI-1 (74), PAI-2 (75), PAI-3 (21) and protease nexin (33), while tcu-PA does. As a consequence, scu-PA is fairly stable in plasma, in contrast to tcu-PA (76), which is unstable.

Plasminogen activation by scu-PA

An extensive discussion has existed in the literature concerning whether or not scu-PA, as a single-chain molecule, is able to activate plasminogen. The problem is, that in a mixture of scu-PA and plasminogen, both plasmin and tcu-PA are generated. After addition of a plasmin inhibitor, which prevents formation of tcu-PA, plasmin is still generated, suggesting that cleavage of scu-PA is not absolutely necessary for activation of plasminogen (77-79). Moreover, plasmin-resistant mutants of scu-PA, with Lys_{158} mutated to Glu_{158}, Gly_{158} or Thr_{158} have a low but significant activity (38,57,80). Similar conclusions have been drawn from *in vitro* studies with mutants in which Lys_{158} was replaced by Val or Met (81), and from animal studies with mutants in which Lys_{158} was replaced by Ala, Glu or Met (the latter in combination with a substitution at position 160) (82).

A sequence of three reactions has been proposed to be involved in plasminogen activation (83,84,78).

$$\text{scu-PA} + \text{plasminogen} \rightarrow \text{scu-PA} + \text{plasmin} \quad \text{I}$$

$$\text{plasmin} + \text{scu-PA} \rightarrow \text{plasmin} + \text{tcu-PA} \quad \text{II}$$

$$\text{tcu-PA} + \text{plasminogen} \rightarrow \text{tcu-PA} + \text{plasmin} \quad \text{III}$$

Each reaction obeys Michaelis-Menten kinetics. Scu-PA activates plasminogen with a much lower k_{cat} than does tcu-PA, but the low catalytic rate constant of scu-PA is partially compensated for by a high affinity for the substrate (low K_m) (83,84). The site(s)

in plasminogen involved in the high-affinity interaction with scu-PA appear to be located within the low molecular weight plasminogen moiety and not in the first four kringle domains (85). The high-affinity interaction has, however, not been found in a kinetic study with a different experimental design (78). Plasmin has a relatively low affinity for scu-PA in the conversion of scu-PA to tcu-PA in reaction II (83,78,84). The plasmin-mediated conversion is enhanced by heparin (86-88,82).

Although most investigators agree with at least some plasminogen activator activity of scu-PA (0.1-1.0% of the activity of tcu-PA) (77,78,89), other investigators doubt whether this activity really represents an intrinsic activity of scu-PA. Petersen et al. showed that scu-PA may indeed have some activity, but considered scu-PA as a genuine proenzyme form of tcu-PA (32). Similarly, Urano et al. questioned whether scu-PA possesses sufficient inherent plasminogen activator activity to play a role under physiological conditions (90).

2.5 Thrombolytic properties

Fibrin-selectivity

Soon after the discovery that u-PA can be obtained as a single-chain molecule, it became clear that scu-PA has a more fibrin-selective action than tcu-PA (72). In other words, lysis of a fibrin clot in a plasma milieu is associated with less systemic effects (e.g. fibrinogen degradation, α_2-antiplasmin consumption) when scu-PA is used instead of tcu-PA. Fibrinolytic efficacy and fibrin-selectivity have been studied with radiolabelled clots immersed in plasma containing the drug (72,81,91-94) and in various experimental animal models, such as open-chest and copper coil coronary artery thrombosis models in baboons (95) or dogs (96-99), a dog femoral artery thrombosis model (100), dog saphenous vein (23,101) and rabbit jugular vein (93,102,103) thrombosis models, and rabbit or dog pulmonary embolus models (72,94).

Different theories have been put forward to explain the fibrin-selective action of scu-PA:

1. Scu-PA is locally activated on the clot surface by fibrin-bound plasmin, which is relatively inaccessible for protease inhibitors. Virtually no activation occurs in the plasma outside the clot, as circulating plasmin rapidly inactivated by α_2-antiplasmin. Tcu-PA is the active species in this theory (72).
2. Direct binding of scu-PA to fibrin could be a mechanism to explain fibrin-selectivity, similar to the mechanism of action of t-PA. Findings that scu-PA binds to fibrin-coated celite (19,30), fibrin-Sepharose (23,25) and even to fibrin clots (24) are in favour of this mechanism. However, significant binding to fibrin clots is not a general

finding (25,31,76, 104), suggesting that such a phenomenon does not play an important role in the fibrin-specific action of scu-PA. Inactivation of scu-PA by thrombin might wrongly suggest that fibrin clot binding does occur if clot binding is estimated only by measuring u-PA in clot supernatants (66). The use of preformed fibrin monomers (25) or ancrod instead of thrombin as clotting agent (66) eliminates this problem.

Recently, a new form of u-PA with fibrin-binding properties has been described (105-107). This form, found in urine, has a molecular weight of about 100,000 and probably represents a complex between tcu-PA and plasminogen activator inhibitor type 3, which is identical with protein C inhibitor.

3. Based on his findings that scu-PA directly activates plasminogen in buffer, but not significantly in plasma in the absence of fibrin, Lijnen et al. (15,79,89) have postulated that plasma contains a competitive inhibitor of scu-PA and that this inhibition is abolished by fibrin. To date, such an inhibitor has not yet been purified and characterized.
4. Scu-PA has a low activity towards native Glu-plasminogen, but a much higher activity towards Lys-plasminogen and fibrin-bound Glu-plasminogen (which has a Lys-plasminogen-like conformation) (76). The difference in sensitivity between free and fibrin-bound Glu-plasminogen explains, at least partially, the fibrin-selectivity of scu-PA. Tcu-PA also has a preference for fibrin-bound plasminogen, but the enhancing effect of fibrin is probably smaller than with scu-PA (108). The effect of the low activity towards Glu-plasminogen in plasma is limited by still undefined protease inhibitors (76), which may be identical with the competitive inhibitor of Lijnen et al. (79).

The latter mechanism is an attractive one to explain the action of scu-PA. However, there is growing evidence, e.g. from studies with plasmin-resistant scu-PA mutants (81,109,89), that amplification of the fibrinolytic process via plasmin-catalyzed activation of scu-PA to tcu-PA is required for rapid *in vitro* clot lysis (a recent study suggests that conversion of scu-PA to tcu-PA plays a less important role for *in vivo* thrombolysis (82)). The aforementioned theory of localized activation may constitute a supplemental mechanism contributing to the fibrin-selectivity of scu-PA.

Synergism of scu-PA and t-PA

Various studies have shown a synergistic effect of scu-PA and t-PA, which might reduce the total amount of fibrinolytic agents required for thrombolytic therapy. An additional potential advantage is that this synergism may increase the fibrin-specificity and thus reduce systemic plasminogen activation.

Synergism has been found in a plasma clot lysis system *in vitro* (110). Other investigators have reported, however, that synergism does not occur in a similar plasma clot lysis system (111,112). Synergism *in vitro* is considered as apparent and can be excluded if the data are analysed by using appropriate dose-response curves, which are log linear for t-PA and sigmoidal for scu-PA (111). The contradiction between the various studies, which may be related to differences in concentrations and ratios of t-PA and scu-PA used, has been discussed extensively (113,114).

Less discussion exists about the occurrence of synergism during fibrinolytic therapy *in vivo*. The beneficial effect of combinations of t-PA and scu-PA has been demonstrated in a jugular vein thrombosis model in the rabbit (115,116,117), in a femoral vein thrombosis model in the dog (117), and in two studies with a small number of patients with acute myocardial infarction (118,119). In one of the latter studies, a dose of approximately one quarter (10 mg t-PA plus 10 mg scu-PA) of the dose currently used for each agent alone appeared to be effective in 7 out of 9 patients (119).

Two studies deal with the mechanisms behind the synergistic effect, either *in vitro* (13,14,120) or *in vivo* (116). Pannell et al. (120) have suggested that t-PA and scu-PA induce fibrinolysis by different but complementary mechanisms. t-PA activates Glu-plasminogen, which is bound to an internal lysine (probably the Aα Lys_{157} (121)), which is already available in undegraded fibrin. By contrast, scu-PA selectively activates Glu-plasminogen, which is bound to COOH-terminal lysine residues. These binding sites are not yet available in undegraded fibrin, but are exposed when fibrinolysis, e.g. initiated by t-PA, takes place. This hypothesis provides an explanation for the typical sigmoidal shape of the scu-PA-induced clot lysis curve versus time and for the synergism between t-PA and scu-PA. Collen et al. (116) investigated the synergistic mode of action of t-PA and scu-PA *in vivo* by using the rabbit jugular vein thrombosis model. They found that a sequential application of first t-PA and then scu-PA results in significant potentiation of thrombolysis, whereas scu-PA followed by t-PA is totally devoid of any synergistic effect. Although these results could be explained by the above-mentioned hypothesis of newly formed COOH-terminal lysine residues in the fibrin clot, the authors believed that another, still unknown, mechanism is responsible for the *in vivo* synergistic effect.

It has not yet been firmly established whether synergism between scu-PA and tcu-PA exists. A preliminary report suggests that the phenomenon does occur during *in vitro* clot lysis (12). Other investigators could not demonstrate synergism in a rabbit model (116). The results of small clinical studies strongly suggest that the addition of a low-dose bolus of tcu-PA increases the efficacy of scu-PA, but no synergism could be claimed from the data (122,123).

Clinical application of scu-PA

Van der Werf et al. were the first to demonstrate the efficacy of scu-PA for coronary thrombolysis in humans (124). Complete reperfusion was obtained with 40 mg of scu-PA administered intravenously, followed by an intracoronary infusion of 20 mg scu-PA, in 5 out of 6 patients with acute myocardial infarction. These results were confirmed and extended in various, often small clinical studies (111,123,125-127). When given without other thrombolytic agents, a dose of at least 70-80 mg of scu-PA is required for optimal reperfusion (123,125,126). The short half-life of scu-PA (117,128,129) necessitates continuous infusions (alpha T½ = 8 min (119)). Systemic plasminogen activation is moderate but less than with tcu-PA, as shown for instance by Trübestein et al. (130). For a more detailed evaluation of the presently available clinical results, the reader is referred to a review of Gulba and Neuhaus (131).

Recently the results of a large (n = 401) randomized double-blind trial of recombinant scu-PA against streptokinase in acute myocardial infarction became available (132). Intravenous scu-PA led to higher patency rate, earlier reperfusion, less disturbance of haemostasis, and fewer bleeding complications than did intravenous administration of streptokinase.

2.6 Concluding remarks

Although some controversies exist about the mechanism of action of scu-PA, its fibrin-selective properties in comparison with tcu-PA have been firmly established. The clinical experience with scu-PA is, however, still limited. Future clinical trials should evaluate the efficacy and bleeding risks of scu-PA in comparison with other thrombolytic agents. New developments will include the exploitation of the synergistic effects of scu-PA with other thrombolytic agents and the construction of mutants of scu-PA by recombinant DNA technology. One simple but promising mutant is low molecular weight scu-PA, which may also be formed by proteolysis in cell cultures. This form shows, at least in laboratory studies, similar properties to normal scu-PA (25,41,92,93,129,133). Other future mutations may, for instance, add fibrin clot binding properties to scu-PA or lengthen its *in vivo* half-life, but these topics are beyond the scope of this review.

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CHAPTER 3

UROKINASE AND ITS COMPLEX WITH PLASMINOGEN ACTIVATOR INHIBITOR-3/PROTEIN C INHIBITOR IN URINE

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Summary

Urokinase-type plasminogen activator (u-PA) occurs either in a single-chain form or in a two-chain form (scu-PA or tcu-PA, respectively). The occurrence of the different forms of u-PA and the formation of u-PA-inhibitor complexes was studied in urine samples from ten healthy donors. Measurement by means of a bio-immunoassay showed that the urine samples contained 307 ± 189 ng/ml u-PA (mean ± S.D.), of which 72 ± 15% occurred in the active two-chain form and 28 ± 15% in the plasmin-activatable single-chain form. SDS-polyacrylamide gel electrophoresis followed by fibrin zymography revealed that fresh urine contained no, or only traces of, u-PA-inhibitor complexes. In contrast to what has previously been published, the bulk of u-PA appeared to be in an uncomplexed 55 kD form. Incubation of urine at 37°C for 8 hours did not lead to the formation of u-PA-inhibitor complexes. However, after rapid removal of the low molecular weight fraction of urine on a gel filtration column a 100 kD complex, consisting of u-PA and plasminogen activator inhibitor-3/protein C inhibitor, was formed during incubation ($t_{½} \approx 45$ min). Complex formation was dependent on stimulation by urinary glycosaminoglycans and only occurred at a low ionic strength, which explains why no complexes were formed in the untreated urine samples of healthy donors.

3.1 Introduction

Urokinase-type plasminogen activator (u-PA) converts plasminogen into plasmin and was first identified in urine (1,2). Initially, purified urinary u-PA preparations appeared to consist of two polypeptide chains (tcu-PA) (3), but it was later shown that urine also contains single-chain u-PA (scu-PA or pro-urokinase) (4,5). Purified scu-PA can be activated to tcu-PA by plasmin, and inactivated by thrombin. The latter reaction is catalyzed by thrombomodulin (6). For reviews on scu-PA, see Gurewich (7); Lijnen et al. (8); and De Munk & Rijken (9). A u-PA inhibitor was found in urine (5,10,11) and has been designated plasminogen activator inhibitor-3 (PAI-3). PAI-3 was shown to be identical to protein C inhibitor (PCI) (12,13). For reviews on PCI, see Geiger (14) and Suzuki et al. (15). Single-chain u-PA does not react with PAI-3, but tcu-PA is rapidly inhibited (11). The complex formation of tcu-PA and PAI-3 is stimulated by heparin (11,16). It has been claimed that tcu-PA present in urine occurs mainly in complex with PAI-3 (5). The tcu-PA-PAI-3 complex binds to fibrin and is probably activated by fibrin (17-19).

In this study we have identified and quantified various plasminogen activators in urine samples from healthy donors and investigated the occurrence and formation of plasminogen activator-inhibitor complexes. Although the physiological role of u-PA in urine is still uncertain, a function in clearance of fibrin deposits in the urinary system has been proposed (1). A role in the prevention of renal stones has also been suggested (20-22). The concentration of u-PA in the urine of patients with impaired renal function is strongly reduced, suggesting that the measurement of fibrinolytic parameters in urine might be of clinical relevance (23).

3.2 Materials and methods

Materials

Two-chain u-PA, used as a marker in SDS-polyacrylamide gel electrophoresis and fibrin zymography, was obtained from Choay (Paris, France). Tissue-type plasminogen activator (t-PA), a gift from Dr. J.H. Verheijen of this laboratory, was purified from a human melanoma cell culture (24,25). Protamine sulphate was from Merck (Darmstadt, Germany) and standard heparin (Thromboliquine) from Organon Teknika (Turnhout, Belgium). The anti-serum against PAI-3/PCI, a gift from Drs. J.C.M. Meijers and B.N. Bouma (University Hospital Utrecht, The Netherlands), was raised in a rabbit by using PCI purified from human plasma (26).

Urine

Individual urine samples, shown in Fig. 1, were collected from ten healthy donors (6 men and 4 women, age 25-57 years) between 8.30 a.m. and 10.30 a.m. and used immediately after adjusting the pH to 7.0 with 1 M NaOH. In all other experiments urine consisted of a pool of freshly voided samples from five donors. After adjusting the pH to 7.0, the pool was centrifuged and used immediately.

Desalting by gel filtration

Aliquots of 250 μl urine were applied on a 5 ml (3.8 x 1.3 cm) Sephadex G-50 (fine) (Pharmacia, Uppsala, Sweden) column in 50 mM Tris/HCl buffer containing 50 mM NaCl and 0.01% (w/v) Tween 80, pH 7.0. Elution was forced by centrifugation at 500 x g for 2 minutes at room temperature (27). The eluted fraction of 250 μl contained the protein fraction.

Bio-immunoassay (BIA)

The BIA is a mixed antigen and activity test (28-30). In brief, PVC microtitre plates were coated with rabbit antibodies against u-PA, and incubated for 2 hours with urine samples, diluted 60-420 times in 15 mM NaCl, 10 mM KH_2PO_4, 0.01% (w/v) Tween 80, 0.1% (w/v) BSA (product number A7030; Sigma, St. Louis, MO, USA), 1000 U/ml Trasylol (Bayer, Leverkusen, Germany), 0.02% NaN_3, 5 mM EDTA, pH 7.4. After washing the plates four times, bound tcu-PA activity was measured with plasminogen and the plasmin substrate D-Val-Leu-Lys-p-nitroanilide (S-2251) essentially as described by Verheijen et al. (31) for t-PA. Background values were obtained by adding an excess of quenching antibodies against u-PA to the samples. The amounts, in ng, of tcu-PA were calculated from a standard curve, made from a tcu-PA preparation, assuming a specific activity of 104,000 IU/mg (32). In parallel measurements, samples were first incubated with plasmin (Kabi, Stockholm, Sweden), at 1 mCU/ml for 60 minutes at 25°C, to activate bound scu-PA and were then assayed for activity as described above, revealing the sum of the amounts of tcu-PA and scu-PA activity.

u-PA activity assay

u-PA activity was measured with plasminogen and S-2251 as follows (materials as described in ref. (31)): samples containing u-PA were mixed with 0.24 μM Lys-plasminogen, 0.12 mg/ml CNBr-fibrinogen and 1.2 mM S-2251 (all final concentrations). The reaction mixtures of 150 μl in 0.1 M Tris/HCl, pH 8.5, containing 0.1% (w/v) Tween 80 were incubated in the wells of a microtitre plate, incubated at 25°C and the absorbance (A) at 405 nm was measured repeatedly. The u-PA activity was expressed as $\Delta A/hr^2$.

SDS-polyacrylamide gel electrophoresis and fibrin zymography

SDS-PAGE was performed according to the method of Laemmli (33) using a 10% polyacrylamide gel. Samples were diluted 1:1 in sample buffer, containing 4% (w/v) SDS, 4% (w/v) glycerol, 0.005% (w/v) bromophenol blue and 0.125 M Tris/HCl, pH 6.8, and were incubated for 30 minutes at room temperature before they were applied to the gel. After electrophoresis the gels were washed with 2.5% (w/v) Triton X-100 and placed over a plasminogen-rich bovine fibrin-agarose layer (34). Where indicated, u-PA and t-PA activities were quenched by incorporating 80 μg/ml rabbit anti-u-PA IgG (35) or 35 μg/ml rabbit anti-t-PA IgG (Organon Teknika, Turnhout, Belgium), respectively, in the fibrin layer.

Immunoprecipitation

Gel-filtered urine was incubated at 37°C for 3 hours to allow u-PA-inhibitor complex formation. The urine was then incubated at 4°C for 4 hours with rabbit anti-PAI-3 serum (1:280 and 1:70) or rabbit anti-u-PA IgG (1.6 and 6.5 μg/ml) or buffer, after adjusting the final serum concentration to 1:70 with nonimmune serum. Antigen-antibody complexes were precipitated by adding swine anti-rabbit IgG (Dakoppats, Glostrup, Denmark; 6.5 mg/ml final concentration) at 4°C overnight. After centrifugation the supernatants were mixed with sample buffer and subjected to SDS-PAGE.

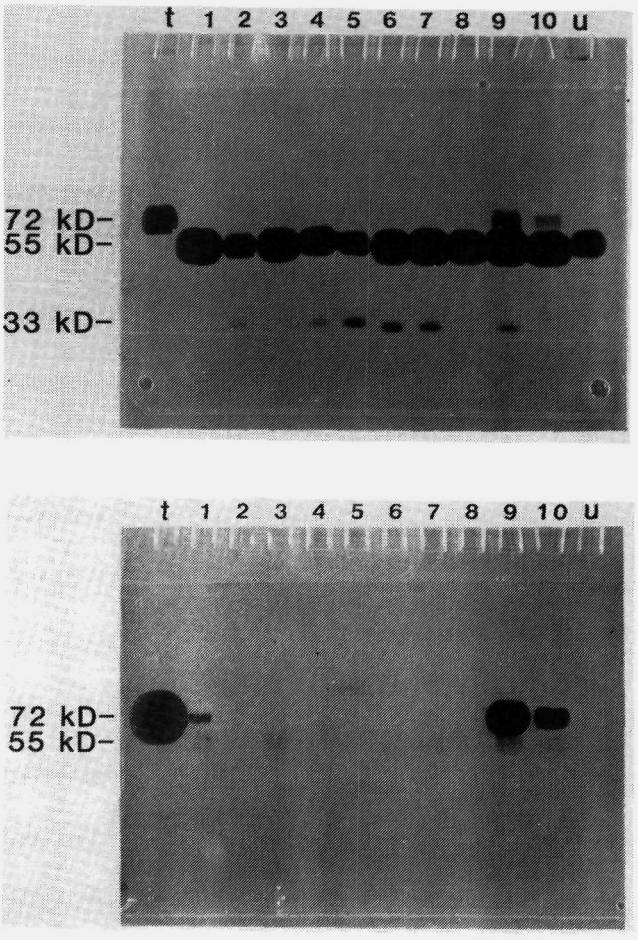

Figure 1. Identification of plasminogen activators in urine. SDS-PAGE of samples of freshly-voided urine from ten different healthy donors followed by fibrin zymography, without (A) and with (B) quenching antibodies against u-PA in the fibrin layer. Lane 1-10 contained various amounts of the different urine samples: 10 μl (lane 2,7,8), 20 μl (lane 1,3,6,9,10) or 40 μl (lane 4,5). Lane t and u contained the following markers: 0.42 IU 72 kD t-PA and 1.0 IU 55 kD tcu-PA, respectively. The photos were taken after 12 hours (A) and 20 hours (B) incubation of the zymograms.

Table 1. tcu-PA and scu-PA concentrations in urine.
Quantification of tcu-PA and scu-PA was done with a bio-immunoassay and results are given in ng/ml. The concentrations of tcu-PA and scu-PA together and of tcu-PA were measured (see Materials and Methods); the concentration of scu-PA was calculated. Subject numbers correspond with lane numbers in Fig. 1.

Subject	scu-PA + tcu-PA	tcu-PA		scu-PA	
	ng/ml	ng/ml	%	ng/ml	%
1	340	263	77	77	23
2	239	189	79	50	21
3	218	161	74	57	26
4	77	68	88	9	12
5	53	41	77	12	23
6	245	119	49	126	51
7	571	538	94	34	6
8	630	357	57	273	43
9	277	193	70	84	30
10	420	218	52	202	48
average	307	215	72	92	28
s.d.	189	146	15	86	15

3.3 Results

Identification and quantification of plasminogen activators

Freshly-voided urine from 10 healthy donors was subjected to SDS-PAGE followed by fibrin zymography. Each sample contained predominantly 55 kD u-PA (Fig. 1A). Six samples also contained tiny amounts of 33 kD u-PA. Three samples contained 72 kD t-PA (Fig. 1B). No u-PA- or t-PA-inhibitor complexes were visible (trace amounts were occasionally observed after prolonged incubation of the zymograms). Both 55 kD and 33 kD u-PA lysis zones were quenched by anti-u-PA IgG in the fibrin layer, while the lysis zones of t-PA remained visible (Fig. 1B). All lysis zones were quenched by a combination of anti-t-PA and anti-u-PA IgG (not shown).

Fibrin zymography does not discriminate between scu-PA and tcu-PA. The concentrations of these two forms in the urine samples shown in Fig. 1 were determined with a bio-immunoassay (Table 1). The sum of the concentrations of tcu-PA and scu-PA

varied between 53 and 630 ng/ml (mean 307 ng/ml). The concentration of tcu-PA alone was 41-538 ng/ml (mean 215 ng/ml). scu-PA concentrations were calculated and showed a range of 9-273 ng/ml (mean 92 ng/ml). These results indicate that 6-51% (mean 28%) of u-PA occurred in the single chain form. No correlation was found between the sum of the concentrations of scu-PA and tcu-PA and the concentration of tcu-PA or the occurrence of 33 kD u-PA.

Complex formation between u-PA and PAI-3

Cieplak & Hart (10) have shown that exhaustive dialysis of urine results in the formation of a stable complex between urokinase and a urinary component with Mr of approximately 40,000. In order to study the formation of the complex in more detail, urine was not dialyzed, but rapidly gel filtered by centrifugation through a Sephadex G-50 column in 50 mM Tris/HCl buffer containing 50 mM NaCl and 0.01% Tween 80, pH 7.0. Urine before and after gel filtration was then incubated at 37°C for 8 hours and serial samples were subjected to SDS-PAGE and fibrin zymography (Fig. 2). Before gel filtration, all time samples showed the same u-PA band at 55 kD. After gel filtration the 55 kD u-PA band decreased, and a new band at about 100 kD appeared at 5 minutes and increased during the following 8 hours of incubation. No significant 33 kD u-PA band appeared within 8 hours.

Although the identity of the Mr ≈ 40,000 urinary component of the 100 kD complex was not established by Cieplak and Hart (10), later studies detected urinary complexes between u-PA and PAI-3/PCI (5,11). Immunoprecipitation experiments showed that the

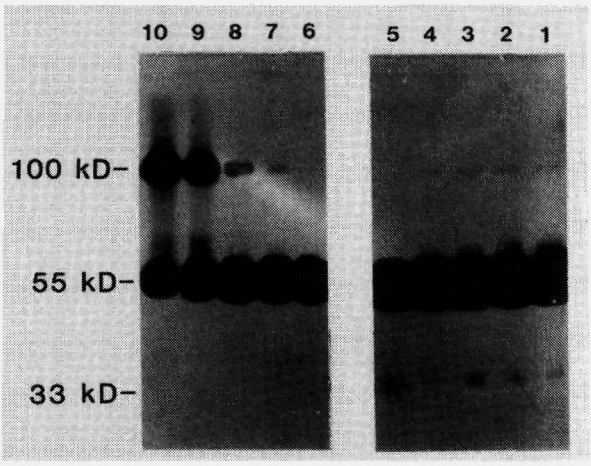

Figure 2. SDS-PAGE followed by fibrin zymography of 20 μl urine before and after rapid gel filtration on a Sephadex G-50 column and during incubation at 37°C. Lane 1-5: urine before gel filtration; samples were taken at t = 0 min (1), t = 5 min (2), t = 30 min (3), t = 2 hr (4), t = 8 hr (5). Lane 6-10: urine after gel filtration; samples were taken at t = 0 min (6), t = 5 min (7), t = 30 min (8), t = 2 hr (9), t = 8 hr (10).

100 kD band which was obtained upon gel filtration and incubation at 37°C disappeared both with an anti-PAI-3 antiserum and with anti-u-PA IgG (Fig. 3), indicating that the 100 kD band obtained in this manner represented u-PA-PAI-3 complex.

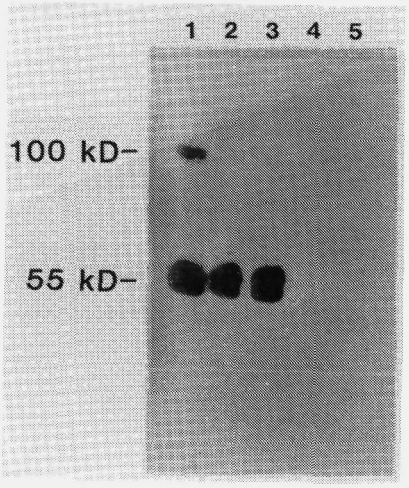

Figure 3. Identification of 100 kD complex as u-PA-PAI-3. SDS-PAGE and fibrin zymography after the removal of u-PA and PAI-3 antigen by immuno-precipitation in urine after gel filtration and 3 hrs incubation at 37°C. Lane 1: control without antibodies, lanes 2 and 3: precipitation with rabbit anti-PAI-3 serum (280-fold and 70-fold diluted, respectively), lanes 4 and 5: precipitation with rabbit anti-u-PA IgG (1.6 and 6.5 μg IgG/ml, respectively).

In order to determine the rate of the decrease of 55 kD u-PA, u-PA activity in urine was measured during incubation at 37°C before and after gel filtration. Before this treatment, u-PA activity remained constant for at least 8 hours at 37°C (not shown).

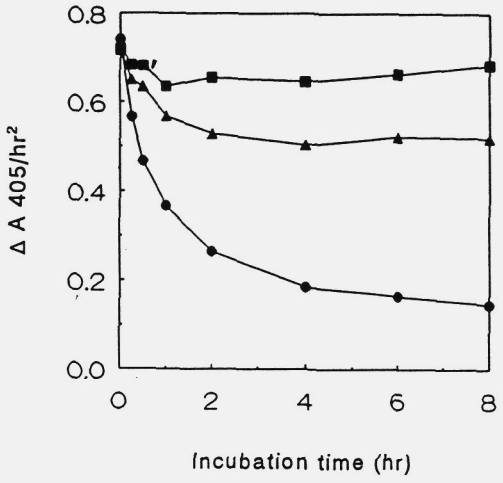

Figure 4. Time course of the decrease of u-PA activity during incubation at 37°C in urine after rapid gel filtration on a Sephadex G-50 column. Aliquots of 5 μl were tested in a plasminogen activation assay and the residual u-PA activities were expressed as the change in absorbance at 405 nm per time square. Freshly-voided urine (•); urine after one freeze-thaw cycle (▲); urine after two freeze-thaw cycles (■).

After gel filtration, a rapid decrease of u-PA activity was found during the incubation, with a half-life of about 45 minutes (Fig. 4). Freezing and thawing of urine stabilized u-PA activity during subsequent gel filtration and incubation at 37°C (Fig. 4), possibly due to denaturation or inactivation of PAI-3.

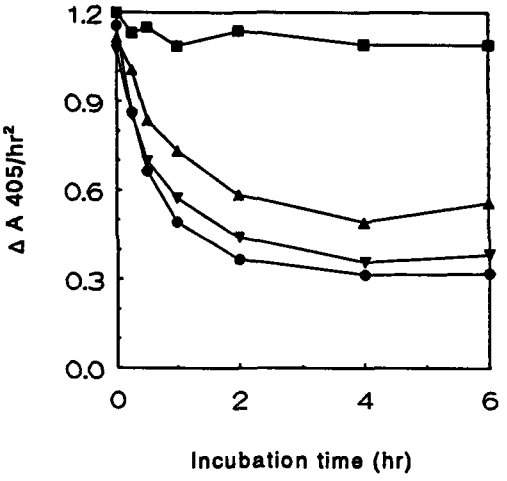

Figure 5. Effect of protamine sulphate on the activity of u-PA. Urine was gel filtered on a Sephadex G-50 column, immediately supplemented with 0 (•), 0.12 (▼), 0.6 (▲) or 1.5 (■) μg/ml protamine sulphate, and incubated at 37°C. Aliquots of 10 μl were tested in a plasminogen activator assay and the residual u-PA activities were expressed as the change in absorbance at 405 nm per time square.

Mechanism of complex formation

As the action of PAI-3/PCI is strongly potentiated by heparin (11,16), the issue was raised as to whether or not urinary glycosaminoglycans are involved in the complex formation between u-PA and PAI-3 in gel-filtered urine. Fig. 5 shows that low concentrations of protamine sulphate (1.5 μg/ml) completely prevented the inhibition of u-PA, suggesting that urinary glycosaminoglycans play an essential role. The potentiation by these compounds was, however, not maximal, as the addition of heparin to gel-filtered urine resulted in a dose-dependent increase in complex formation (Fig. 6). High concentrations of heparin (above 1.0 IU/ml) inhibited the formation of the 100 kD complex.

To find out why u-PA did not react with PAI-3 in untreated urine, gel-filtered urine was mixed (1:1) with the salt fraction of urine, obtained by dialysing 1 ml water in 200 ml urine in a membrane permeable for molecules up to 10 kD. Incubation of this mixture at 37°C for 24 hours did not result in complex formation (not shown). A major component of the salt fraction of urine is NaCl. Fig. 7 shows that the loss of u-PA activity during incubation of gel-filtered urine was completely prevented by adding 50 mM NaCl to the gel-filtered urine, which already contained 50 mM NaCl. Addition of salts other than NaCl (NH_4Cl, KCl, Na_2SO_4, $NaHCO_3$, Na_2HPO_4) was at least equally effective (not shown). Adding 500 mM urea did not prevent the loss of u-PA activity during incubation.

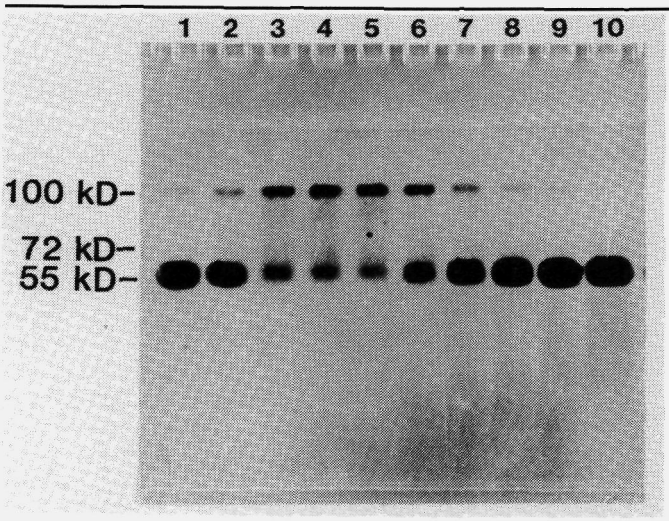

Figure 6. Effect of heparin on the formation of the u-PA-PAI-3 complex (100 kD) in gel-filtered urine. SDS-PAGE and fibrin zymography of gel-filtered urine before (lane 1) and after 30 min incubation at 37°C in the presence of 0 (lane 2), 0.016 (lane 3), 0.063 (lane 4), 0.25 (lane 5), 1.0 (lane 6), 4.0 (lane 7), 16 (lane 8), 64 (lane 9) or 256 (lane 10) IU/ml added heparin.

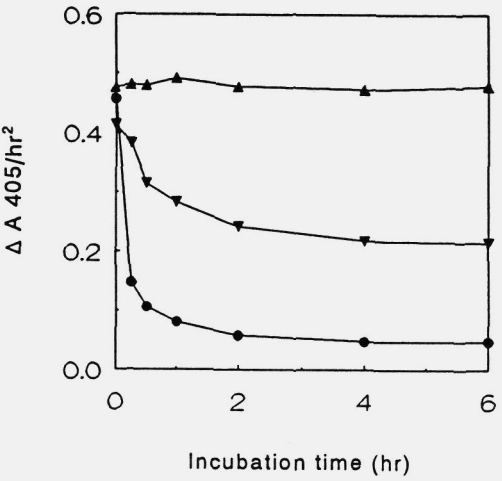

Figure 7. Effect of NaCl on the stability of u-PA. Urine was gel filtered on a Sephadex G-50 column (in 50 mM Tris-HCl buffer, containing 50 mM NaCl and 0.01% Tween 80, pH 7.0), immediately supplemented with 0 (•), 25 (▾) or 50 (▲) mM NaCl, and incubated at 37°C. Aliquots of 3 μl were tested in a plasminogen activator assay and the residual u-PA activities were expressed as the change in absorbance at 405 nm per time square.

3.4 Discussion

This study deals with the identification and quantification of various plasminogen activators and activator-inhibitor complexes in urine. A systematic study of the concentrations of different u-PA forms is lacking in the literature. By using a recently

developed bio-immunoassay, we found an average of about 300 ng/ml active u-PA (tcu-PA) and plasmin-activatable u-PA (scu-PA) in freshly-voided urine from 10 healthy donors. This is somewhat higher than earlier reported for pools of urine (50-200 ng/ml (4,5,36)), and is possibly due to the early time of the day at which urine was collected in this study. The percentage of scu-PA in the separate urine samples varied considerably from 6-51% (average 28%). It is still unknown which enzyme is responsible for the activation of scu-PA to tcu-PA. Potential activators are plasmin, plasma kallikrein, trypsin, cathepsin B and cathepsin L (37).

In fresh urine we found predominantly 55 kD u-PA and only traces of 33 kD u-PA. In several commercial tcu-PA preparations a large portion is in the 33 kD form (38), probably derived from 55 kD u-PA during the isolation and purification. We did not find formation of 33 kD u-PA during incubation of urine for 8 hours at 37°C (Fig. 2), but we noticed some conversion after incubation for 18 hours or more (unpublished observation). Urinary 33 kD u-PA begins at the aminoterminus with Lys 136 (39) and may be produced by plasmin or urokinase itself. It is unlikely that the recently described metalloproteinase Pump-1 (40) is involved in the formation of urinary 33 kD u-PA, as Pump-1 cleaves 55 kD u-PA between Glu 143 and Leu 144, resulting in a low molecular weight form with Leu 144 as aminoterminus.

The occurrence of t-PA activity (35) and t-PA antigen (41) in urine has previously been demonstrated. In the present study a 72 kD t-PA band was detected by SDS-PAGE and fibrin zymography (Fig. 1). The activator was found in three out of ten urine samples. The type of cell from which urinary t-PA originated remains to be established.

Only traces of u-PA-inhibitor complexes, if at all, were detected with SDS-PAGE followed by fibrin zymography, either in freshly voided urine samples or in samples after 0-8 hours incubation at 37°C. Complex formation was, however, demonstrated after rapidly removing the low molecular weight fraction of urine by centrifugation through a Sephadex G-50 column. The complex consisted of u-PA and PAI-3, as shown by immunoprecipitation with both anti-PAI-3 and anti-u-PA antibodies (Fig. 3), which agrees with data reported by Stump et al. (5). Our results confirm and extend the results of Cieplak & Hart (10), who found a 95 kD u-PA-complex after exhaustive dialysis of urine. Our rapid gel filtration procedure made it possible to determine the time-dependence of the complex formation, both qualitatively by SDS-PAGE (Fig. 2) and quantitatively by the u-PA activity assay (Fig. 4). The latter results showed that 50% of tcu-PA activity in gel-filtered urine was inhibited in about 45 min. The procedure followed also enabled us to study the mechanism of complex formation induced by gel filtration or dialysis. Complex formation in gel-filtered urine was strongly inhibited by protamine sulphate (Fig. 5), suggesting that the reaction was dependent on the stimulating effect of

glycosaminoglycans in urine. These components occur in urine at a concentration of about 10 μg/ml and consist largely of chondroitin sulphate (42), a type of glycosaminoglycan which is able to stimulate the action of PAI-3/PCI (43). The stimulation of the reaction in gel filtered urine was not maximal, as addition of heparin further accelerated complex formation (Fig. 6). High concentrations of heparin inhibited the reaction, which is indicative of a template model in which both u-PA and the inhibitor have to bind to the same glycosaminoglycan molecule for optimal reaction conditions (44).

Addition of small amounts of NaCl (Fig. 7) or other salts to gel-filtered urine completely blocked the reaction between u-PA and PAI-3. This observation explains why complex formation did not occur in untreated urine. The strong salt sensitivity of the reaction may be related to the nature of the stimulatory interaction of the glycosaminoglycans with both u-PA and PAI-3, which is most likely electrostatic. It is interesting to note that heparin stimulation of the reaction between u-PA and plasminogen also strongly depends on the ionic strength (45), just as the heparin stimulation of the inhibition of protein C by PAI-3/PCI (46).

The apparent absence of u-PA-inhibitor complexes in untreated urine samples and the presence of free tcu-PA seem to contrast with a report by Stump et al. (5), who concluded that the bulk of tcu-PA in urine is complexed with PAI-3. The authors suggested that the relatively high content of free tcu-PA in commercial u-PA preparations from urine may be ascribed to the conversion of scu-PA to tcu-PA or to dissociation of tcu-PA-PAI-3 complexes during the purification process (5). The present study suggests, however, that the tcu-PA-PAI-3 complex observed by Stump et al. has been formed largely during the purification procedure used, possibly after the zinc chelate Sepharose, which binds both u-PA and PAI-3.

It is still not known whether PAI-3 plays a regulatory role for urinary u-PA activity *in vivo*. The apparent absence of complexes in urine samples from healthy donors in the present study does not exclude the possibility that u-PA inhibition could occur in pathological conditions. A study of putative complexes in urine of various patients by SDS-PAGE and fibrin zymography could answer this question. In addition, inhibition of u-PA by PAI-3 may occur locally, stimulated by glycosaminoglycans present on cell surfaces, e.g. on epithelial kidney cells (47).

In conclusion, the present study affords the following contributions. 1. An explanation both for the inhibition of u-PA by PAI-3 during dialysis of urine (10) and for the remarkable observation of Stump et al. (5) that only tcu-PA-PAI-3 complexes could be purified from urine but not free tcu-PA. 2. A practical suggestion for assays of u-PA activity in urine. If urine samples are dialyzed before the assay, the ionic strength of the

dialysis buffer should be sufficiently high to prevent inhibition of tcu-PA by PAI-3. 3. A deeper insight into the components which might regulate the plasminogen activating potential in urine, including single-chain and two-chain u-PA, t-PA, PAI-3/PCI and glycosaminoglycans.

3.5 Acknowledgements

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CHAPTER 4

INTERACTION OF PLASMINOGEN ACTIVATORS AND PLASMINOGEN WITH HEPARIN. EFFECT OF IONIC STRENGTH.

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Summary

In order to define the possible effects of heparin on the fibrinolytic system under physiological conditions, we studied the interactions of this drug with plasminogen and its activators at various ionic strengths. As reported in recent literature, heparin stimulated the activation of Lys-plasminogen by high molecular weight (HMW) and low molecular weight (LMW) two-chain urokinase-type plasminogen activator (u-PA) and two-chain tissue-type plasminogen activator (t-PA) 10- to 17-fold. Our results showed, however, that this stimulation only occurred at low ionic strength and was negligible at a physiological salt concentration. Direct binding studies were performed using heparin-agarose column chromatography. The interaction between heparin and Lys-plasminogen appeared to be salt sensitive, which explains at least in part why heparin did not stimulate plasminogen activation at 0.15 M NaCl. The binding of u-PA and t-PA to heparin-agarose was less salt sensitive. Results were consistent with heparin binding sites on both LMW u-PA and the amino-terminal part of HMW u-PA. Single-chain t-PA bound more avidly than two-chain t-PA. The interactions between heparin and plasminogen activators can occur under physiological conditions and may modulate the fibrinolytic system.

4.1 Introduction

Heparin is a potent anti-thrombotic drug. It inhibits the coagulation pathway, but also has other effects on the haemostatic system. For instance, heparin and related glycosaminoglycans may stimulate fibrinolysis, both by activating the intrinsic fibrinolytic system (factor XII and kallikrein) and by inducing the release of fibrinolytic enzymes, such as tissue-type plasminogen activator (t-PA), from the vascular wall (1). In addition, various recent studies in purified systems have shown that heparin strongly stimulates the activation of plasminogen by t-PA as well as by urokinase-type plasminogen activator (u-PA) (2-10). It is possible to prepare heparin fractions which have intact anticoagulant properties, but are devoid of fibrinolytic stimulating properties, indicating that distinct sites in heparin are involved in the two activities (11,12). As heparin is administered concomitantly with plasminogen activators during thrombolytic therapy following myocardial infarction, several authors have suggested that the stimulatory action of heparin might enhance systemic plasminogen activation and thus reduce clot selectivity. However, other *in vitro* studies have shown that heparin does not enhance t-PA-induced plasminogen activation in plasma or in blood (13,14). While studying this apparent

discrepancy, we made a striking observation that all authors who have shown the stimulating properties of heparin have used buffers with a low, sub-physiological ionic strength (2-12). As heparin has many negatively charged groups, ionic bonds are likely to occur in heparin-protein interactions. We have studied here the effect of the ionic strength on the stimulation of heparin of plasminogen activation in purified systems and, in addition, on binding experiments with heparin-agarose in order to obtain a better understanding of the characteristics of the interactions between heparin and various fibrinolytic components. These components included high molecular weight (HMW) and low molecular weight (LMW) forms of both single-chain and two-chain u-PA (scu-PA and tcu-PA, respectively), single-chain and two-chain t-PA (sct-PA and tct-PA, respectively), and Lys-plasminogen.

4.2 Materials and methods

Materials

Standard heparin (sodium salt) was obtained as a solution of 5000 IU/ml (Thromboliquine) from Organon Teknika (Turnhout, Belgium). Heparin-agarose was bought as heparin-Sepharose CL-6B from Pharmacia (Uppsala, Sweden); plasmin, S-2251 (D-valyl-L-leucyl-L-lysine-p-nitroanilide) and S-2444 (L-pyroglutamyl-glycyl-L-arginine-p-nitroanilide) from KabiVitrum (Stockholm, Sweden), and D-valyl-L-phenylalanyl-L-lysine chloromethyl ketone from Calbiochem (San Diego, Ca, USA). Urinary HMW tcu-PA (Ukidan) was from Serono (Aubonne, Switzerland), tissue culture (human kidney cells) LMW tcu-PA (Abbokinase) from Abbott Laboratories (North Chicago, Il, USA), while tissue culture (human kidney cells) HMW and LMW scu-PA were gifts from Abbott Laboratories (15). In one experiment LMW scu-PA was converted into LMW tcu-PA with plasmin (see legend of Fig. 8). Single-chain t-PA and tct-PA were purified from cultured melanoma cells (16,17) and kindly provided by Dr. J.H. Verheijen of this laboratory. Lys-plasminogen was donated by KabiVitrum (Stockholm, Sweden).

Assay of plasmin

The effects of heparin and ionic strength on the amidolytic activity of plasmin were determined in wells of microtitration plates containing 150 μl mixtures of 8.4 nM (19 mCU/ml) plasmin and 1.2 mM S-2251 in 50 mM Tris-HCl, pH 7.2, containing 0.01% Tween 80. The initial rate of hydrolysis at 25°C was determined by repeated measurements of the absorbance at 405 nm in a Titertek multiskan (Eflab Oy, Finland) and expressed as $\Delta A/h$.

Assay of plasminogen activation

The effects of heparin and ionic strength on the activation of plasminogen were determined in wells of microtitration plates containing 150 μl mixtures of the indicated plasminogen activator, 0.24 μM Lys-plasminogen, and 1.2 mM S-2251. The plasminogen activators were 3.0 IU/ml HMW tcu-PA, 4.2 IU/ml LMW tcu-PA (units from the manufacturers) and 21 ng/ml tct-PA. The buffer consisted of 50 mM Tris-HCl, pH 7.2, containing 50 mM NaCl and 0.01% Tween 80. The initial rate of plasminogen activation at 25°C was determined by repeated measurements of the absorbance at 405 nm and expressed as $\Delta A/h^2$ (18).

Heparin binding experiments

The effect of the ionic strength on heparin binding was studied by heparin-agarose affinity chromatography at 4°C. One ml samples, containing the indicated amounts of plasminogen activator or Lys-plasminogen in 50 mM Tris-HCl, pH 7.2, containing 0.01% Tween 80 (equilibration buffer) were applied to a 4.5 ml heparin-agarose column (3.5 x 1.3 cm). After washing the column with 18 ml equilibration buffer, a linear NaCl gradient (total volume 80 ml) from 0-1.0 M NaCl was applied. Fractions of 1.8 ml were collected at a flow rate of 7.2 ml/h (the fraction volume in Fig. 6 was 2.0 ml). The gradient was determined by measuring the conductivity of the fractions. The positions of the proteins were determined by measuring the activities of tcu-PA and scu-PA (after activation with plasmin) with S-2444 (15), of sct-PA and tct-PA according to Verheijen et al. (18) and of Lys-plasminogen according to Friberger et al. (19). The total recoveries of the proteins ranged from 84 to 108%.

SDS-polyacrylamide gel electrophoresis (PAGE)

SDS-PAGE was performed according to Laemmli (20), using a 10% polyacrylamide gel and was followed by fibrin zymography (21).

4.3 Results

Effect of the ionic strength on plasminogen activation in the presence of heparin

Plasminogen activation was studied in mixtures of a plasminogen activator (HMW tcu-PA, LMW tcu-PA or tct-PA), Lys-plasminogen, heparin and the plasmin substrate S-2251. The direct effects of variations in ionic strength on the activity of plasmin on S-2251 are shown in Fig. 1. In the absence of heparin the NaCl concentration did not significantly affect the activity of plasmin. In the presence of heparin, however, the activity of plasmin

was strongly suppressed at low NaCl concentrations and was essentially normalized at 100 mM NaCl. Plasminogen activation experiments were carried out in 0.05 M Tris-HCl, pH 7.2, containing 0.01% Tween 80 and at least 50 mM NaCl.

Fig. 2 shows the effect of the heparin concentration on the plasminogen activation by HMW and LMW tcu-PA (from urine and tissue culture, respectively) at 50 mM NaCl. Both forms of tcu-PA were strongly stimulated (16- and 17-fold, respectively). The LMW form required a four times higher heparin concentration than the HMW form for half-maximal stimulation. At heparin concentrations above 50 IU/ml, the activities of HMW and LMW tcu-PA decreased again. This decrease was also observed with heparin dialyzed against the incubation buffer to eliminate salts or low molecular weight contaminants (not shown). The bell-shaped curves could be explained by a template mechanism for the activation of plasminogen in the presence of heparin (22). A concentration of 10 IU/ml heparin was chosen for the next experiments to achieve optimal stimulation of tcu-PA.

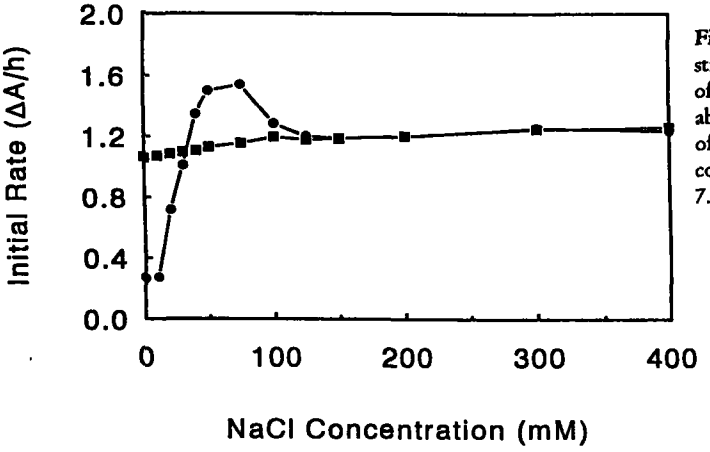

Figure 1. *The effect of the ionic* strength on the amidolytic activity of plasmin on S-2251, both in the absence (■) and in the presence (•) of 10 IU/ml heparin. The buffer consisted of 50 mM Tris/HCl, pH 7.2 containing 0.01% Tween 80.

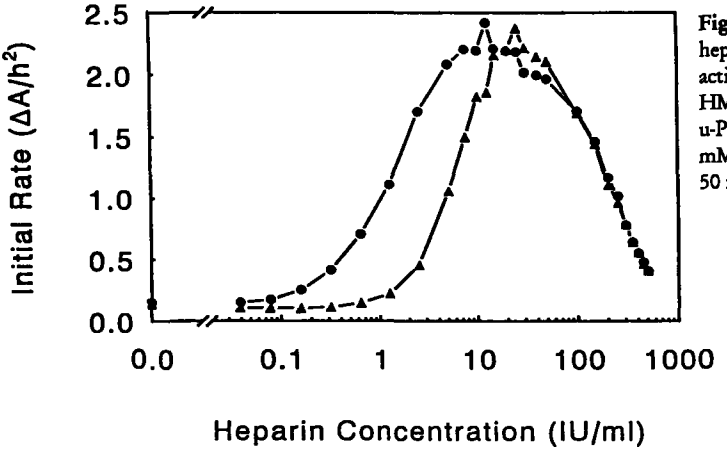

Figure 2. The effect of the heparin concentration on the activation of Lys-plasminogen by HMW (•) and LMW (▲) two-chain u-PA. The buffer consisted of 50 mM Tris/HCl, pH 7.2, containing 50 mM NaCl and 0.01% Tween 80.

The effect of the ionic strength on the activation of Lys-plasminogen by HMW and LMW tcu-PA is shown in Fig. 3. In the absence of heparin, addition of extra NaCl to the Tris-buffer, containing already 50 mM NaCl, slightly suppressed the activities of the two activators. In the presence of heparin, however, the activities rapidly dropped to the levels of the unstimulated activators. The stimulation by heparin was entirely eliminated upon addition of 75 and 100 mM NaCl to LMW and HMW tcu-PA, respectively (final NaCl concentrations 125 and 150 mM).

The effect of the ionic strength on tct-PA and the stimulation of tct-PA by heparin was studied as for tcu-PA. Fig. 4 shows a bell-shaped curve, obtained with increasing heparin concentrations in the Tris-buffer, containing 50 mM NaCl, pointing to a template mechanism.

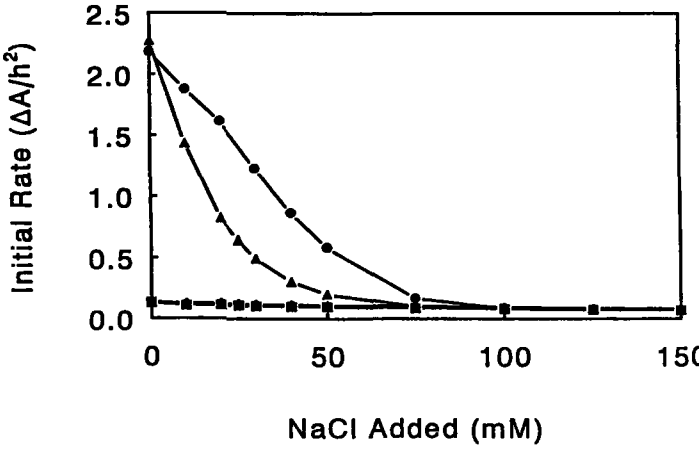

Figure 3. The effect of the ionic strength on the activation of Lys-plasminogen by HMW (■) and LMW (◆) two-chain u-PA in the absence of heparin, and by HMW (•) and LMW (▲) two-chain u-PA in the presence of 10 IU/ml heparin. The ionic strength was varied by adding NaCl to the buffer consisting of 50 mM Tris/HCl, pH 7.2, containing 50 mM NaCl and 0.01% Tween 80.

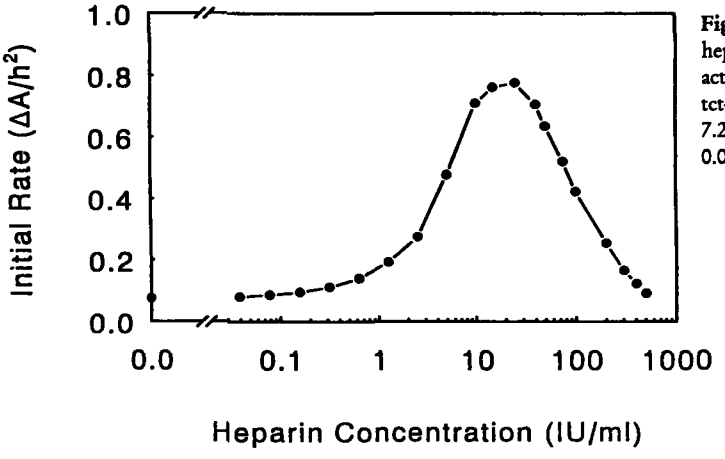

Figure 4. The effect of the heparin concentration on the activation of Lys-plasminogen by tct-PA in 50 mM Tris/HCl, pH 7.2, containing 50 mM NaCl and 0.01% Tween 80.

The maximal stimulation was about 10-fold. The effect of the ionic strength was determined in the presence and the absence of 10 IU/ml heparin. Fig. 5 shows that the stimulatory effect of heparin was entirely eliminated upon the addition of 75 mM NaCl (final NaCl concentration 125 mM).

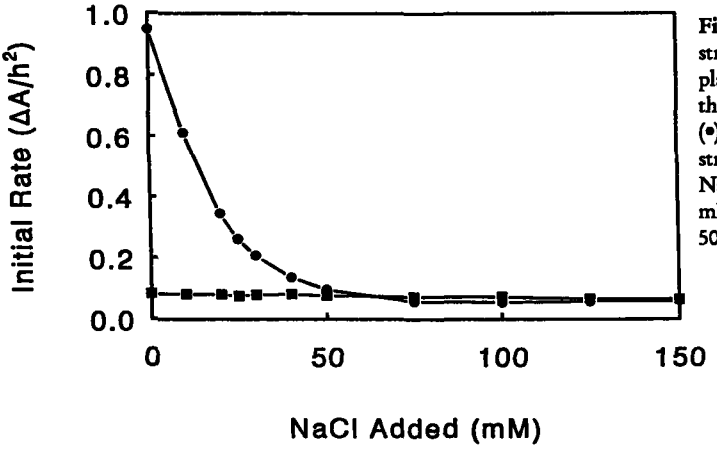

Figure 5. The effect of the ionic strength on the activation of Lys-plasminogen by two-chain t-PA in the absence (■) and in the presence (•) of 10 IU/ml heparin. The ionic strength was varied by adding NaCl to the buffer consisting of 50 mM Tris/HCl, pH 7.2, containing 50 mM NaCl and 0.01% Tween 80.

Binding of the fibrinolytic components to heparin-agarose

The salt sensitivity of the interactions of u-PA, t-PA and Lys-plasminogen with heparin was also studied in direct binding experiments. These experiments were carried out with the use of heparin-agarose column chromatography in 0.05 M Tris-HCl, pH 7.2, 0.01% Tween 80 at 4°C. All fibrinolytic components appeared to be bound completely under these experimental conditions (Figs. 6-9). The strength of the binding was evaluated by determining at which NaCl concentration the components were eluted in a linear gradient from 0 to 1.0 M NaCl (results summarized in Table 1).

HMW tcu-PA from human urine yielded a main peak at 0.42 M NaCl and a small one at 0.20 M (Fig. 6). SDS-PAGE followed by fibrin zymography (Fig. 6, inset) showed that the latter represented a LMW tcu-PA contaminant, implying that HMW tcu-PA bound more tightly to heparin-agarose than LMW tcu-PA. A trace component was observed in fraction number 26 revealing both an intermediate molecular weight and an intermediate affinity to heparin-agarose.

HMW and LMW scu-PA, purified from the culture medium of human kidney cells, eluted at 0.40 and 0.29, respectively (Fig. 7), indicating that the integrity of the N-terminal part of the molecule is also of importance for heparin binding in case of the single-chain forms of u-PA.

Table 1. Binding of fibrinolytic proteins to heparin-agarose columns.
The positions of the protein peaks eluted in a NaCl gradient from 0-1.0 M (Figs. 6-9) are indicated.

Protein	Molecular Form	Elution (M)
Urinary u-PA	HMW tcu-PA	0.42
	LMW tcu-PA	0.20
Tissue Culture u-PA	HMW scu-PA	0.40
	LMW scu-PA	0.29
	LMW tcu-PA	0.25
Melanoma t-PA	sct-PA	0.45
	tct-PA	0.36
Plasminogen	Lys-form	0.19

Comparison of Figs. 6 and 7 suggests that HMW tcu-PA and HMW scu-PA eluted at a similar NaCl concentration from heparin-agarose (at 0.42 and 0.40 M, respectively), but that LMW tcu-PA (from urine) eluted earlier than LMW scu-PA (from tissue culture) (at 0.20 and 0.29 M, respectively). In order to study the importance of the single-chain structure, LMW scu-PA was activated with plasmin, mixed with untreated LMW scu-PA and applied to the heparin-agarose column. Fig. 8 shows that LMW tcu-PA and LMW scu-PA eluted at 0.25 and 0.29 M NaCl, respectively, thus indicating that the two-chain form of LMW u-PA had a slightly lower affinity to heparin-agarose than the single-chain form. The elution behaviour of tissue culture LMW tcu-PA (0.25 M) differed, however, from that of urinary LMW tcu-PA (0.20 M).

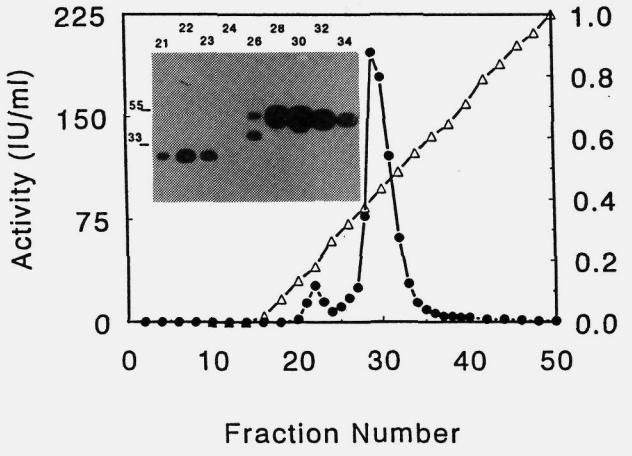

Figure 6. Heparin-agarose chromatography of HMW two-chain u-PA (2000 IU ≈ 20 μg) from urine (•) in a NaCl gradient from 0-1.0 M (△). The inset shows the results of SDS-PAGE and fibrin zymography of fractions 21-34. Molecular weight markers of 55,000 and 33,000 are indicated.

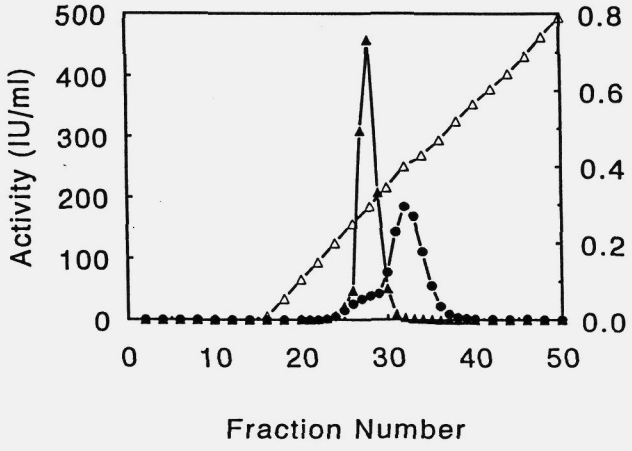

Figure 7. Heparin-agarose chromatography of 20 μg (1600 IU) HMW (•) and 20 μg (1750 IU) LMW (▲) single-chain u-PA from tissue culture. The results of two separate runs are compiled in one graph. Measurements of the amidolytic activity on S-2444 without *prior* activation of scu-PA indicated that no conversion of single-chain to two-chain u-PA occurred on the column (not shown).

Fig. 9 shows the elution profiles of single-chain and two-chain t-PA as well as the profile of Lys-plasminogen. Although the t-PA peaks were broader than the u-PA peaks in Figs. 6-8 their positions were roughly comparable to those of HMW scu-PA and tcu-PA. Fig. 9 indicates, however, that tct-PA eluted earlier (at 0.36 M) than sct-PA (at 0.45 M), pointing to a higher affinity of the single-chain form. Lys-plasminogen eluted as a sharp peak at a relatively low NaCl concentration of 0.19 M.

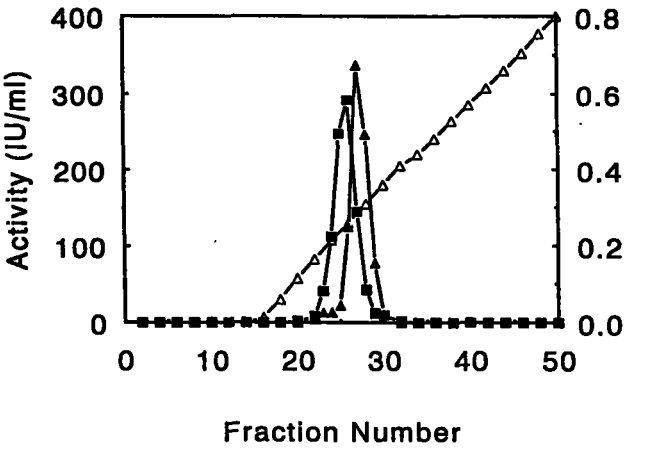

Figure 8. Heparin-agarose chromatography of a mixture of 10 μg LMW single-chain u-PA from tissue culture (▲) and 10 μg of the corresponding two-chain u-PA (■). The latter form was prepared as follows: 10 μg LMW scu-PA was incubated with 50 mCU (22 pmoles) of plasmin for 20 min at 37°C in a volume of 50 μl. The excess of plasmin was then blocked with 2 μM D-valyl-L-phenylalanyl-L-lysine chloromethyl ketone. LMW tcu-PA in the fractions was assayed directly with S-2444, whereas the sum of LMW tcu-PA and LMW tcu-PA was measured after activation with plasmin. LMW scu-PA in the fractions was then calculated by subtraction.

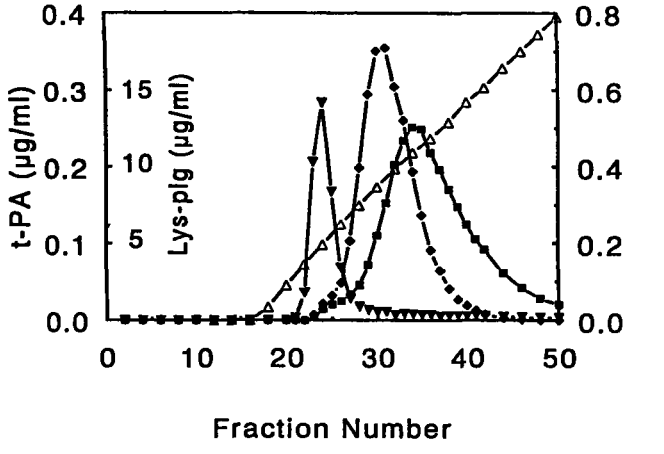

Figure 9. Heparin-agarose chromatography of 5 μg sct-PA (■), 5 μg tct-PA (◆) and 100 μg Lys-plasminogen (▼). The results of three separate runs are compiled in one graph.

4.4 Discussion

Thrombolytic therapy using t-PA and other fibrin-selective agents requires heparin as an adjunct in order to prevent new fibrin formation and rethrombosis (23). On the other hand, experiments in purified systems have shown that heparin enhances plasminogen activation by t-PA and u-PA, which may increase systemic plasmin generation and decrease plasmin generation on the fibrin clot surface, thus impairing fibrin-specific thrombolysis (2-12). However, all latter studies have been performed at sub-physiological ionic strength. The present study evaluated the effect of the ionic strength on the

plasminogen activation and showed that stimulation by heparin is very salt sensitive and negligible at physiological ionic strength. This indicates that heparin should not longer be considered as an agent which stimulates t-PA or u-PA-induced plasminogen activation. The results provide an explanation as to why no heparin stimulation could be observed in plasma and citrated blood (13,14) and suggest that heparin does not enhance systemic plasminogen activation during thrombolytic therapy with t-PA or u-PA.

Stimulation of plasminogen activation was studied in a purified system containing a plasminogen activator, Lys-plasminogen and the plasmin substrate S-2251. At 50 mM NaCl the stimulation by heparin, at an optimal dose of 10 IU/ml, varied between 10 and 17 times for HMW tcu-PA, LMW tcu-PA and tct-PA, which confirmed literature data obtained at a low ionic strength (2-12). At 0.15 M NaCl stimulation by 10 IU/ml heparin was, however, not longer detectable.

The bell-shaped curves obtained upon varying the heparin concentration (Figs. 2 and 4) indicated that a template model (22) might be applicable for all plasminogen activators, implying cyclic ternary complexes between heparin, Lys-plasminogen and the plasminogen activator. For tct-PA and HMW tcu-PA (not for LMW tcu-PA) this model has been supported by direct binding data at low ionic strength (0.1 M Tris-HCl, pH 7.4), showing a Kd of 2.1 μM for the Lys-plasminogen-heparin interaction, a Kd of 1.9 μM for the tct-PA-heparin interaction and a Kd of 2.9 μM for the HMW tcu-PA-heparin interaction (2). The interaction between Glu-plasminogen and heparin is much weaker (Kd = 120 μM) (2). In order to investigate whether the salt sensitivity of the heparin stimulation could be ascribed to a high salt sensitivity of one or more of the separate interactions, binding studies were performed using heparin-agarose. The results (Table 1) showed that all heparin-protein complexes could be disrupted with NaCl-concentration between 0.19 and 0.45 M. The weakest complex was that with Lys-plasminogen, as the latter protein eluted at 0.19 M NaCl. This interaction occurs in all plasminogen activation experiments and may explain a significant portion of the salt sensitivity of the heparin stimulation. However, as the experimental conditions of the plasminogen activation experiments and the binding studies were not identical (different heparin preparations, soluble versus immobilized heparin, 25°C versus 4°C), it is difficult to conclude quantitatively to what extent the salt sensitivity of the separate interactions explains the salt sensitivity of the heparin stimulation. Other parameters, such as a salt-dependent conformation of heparin (24), may be important for an optimal co-localization of plasminogen and its activators on a heparin molecule.

Although it is now clear that heparin is not likely to significantly accelerate the plasminogen activation reaction *in vivo*, plasminogen activator interactions with heparin and related glycosaminoglycans may well be important, either to elevate local

plasminogen activator concentrations e.g. on cell surfaces (25) or to modulate the reactivity of plasminogen activators in various physiological processes. An example is the enhanced sensitivity of scu-PA in the presence of heparin for activation by plasmin (26) and for inactivation by thrombin (27). Therefore we have exploited the affinity chromatography with heparin-agarose to further characterize the heparin-binding sites on the plasminogen activator molecules.

The first clear observation was the difference in heparin binding between HMW u-PA and LMW u-PA, lacking the growth factor and kringle domains, observed both with urinary two-chain forms and with tissue culture single-chain forms. The stronger binding of HMW tcu-PA correlated well with the four times lower heparin concentration required for half-maximal stimulation (Fig. 2) and with the lower salt sensitivity of this stimulation (Fig. 3). The observed difference between HMW u-PA and LMW u-PA can be explained by a recently described binding site in the kringle domain in the amino-terminal part of HMW u-PA, containing a consecutive sequence of three arginine residues (25).

It should be noted, however, that LMW scu-PA and LMW tcu-PA also bound to heparin-agarose. The significance of this binding is illustrated by the observations that the maximal stimulation factors for HMW and LMW tcu-PA in the plasminogen activation assay were almost equal and that the stimulation of LMW tcu-PA also obeyed the template model (Fig. 2), implying an essential interaction between heparin and LMW tcu-PA. HMW u-PA thus may have at least two binding sites for heparin.

LMW tcu-PA from urine bound less strongly to heparin-agarose than did LMW tcu-PA from tissue culture; they were eluted at 0.20 and 0.25 M NaCl, respectively (Table 1). The protein structures of urinary and tissue culture LMW tcu-PA begin with Lys 136 and Leu 144, respectively (28-30), suggesting that a small difference in amino-terminus may affect the affinity for heparin. Recently we have observed a significant effect of variations in amino-terminus of LMW scu-PA with respect to its sensitivity for inactivation by the thrombin-thrombomodulin complex (31), where an interaction between scu-PA and the glycosaminoglycan moiety of thrombomodulin may play a role (32).

Because the chain structure (single-chain versus double-chain) of u-PA and t-PA probably influences the conformation of the molecules, the effect of the chain structure on heparin binding was studied. Single-chain u-PA bound slightly better than tcu-PA when the LMW forms were compared (Fig. 8). This difference was not detectable in the more tightly binding HMW forms. Single-chain t-PA showed a significantly higher affinity for heparin than tct-PA. As heparin binding sites on t-PA have been localized on the finger and kringle 2 domains (33), which also harbour the fibrin binding sites (34,35), our observation is consistent with the observation that sct-PA binds somewhat more

avidly to fibrin than tct-PA (36,37). Single-chain t-PA also binds better than tct-PA to fibronectin (38).

In conclusion, the most striking results of the binding experiments were the demonstration of heparin binding of both HMW and LMW u-PA, consistent with binding sites on both LMW u-PA and the amino-terminal part of HMW u-PA, and the difference in affinity between single-chain and two-chain t-PA. Although the interactions between heparin and plasminogen activators did not result in enhanced plasminogen activation, they may occur and may play a regulatory role under physiological conditions.

After completion of this manuscript, Young et al. (39) demonstrated the strong modulating effects of ionic strength, divalent cations and chloride on the stimulation of Glu-plasminogen activation by heparin. No binding studies explaining the effects were included.

4.5 Acknowledgements

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CHAPTER 5

ACCELERATION OF THE THROMBIN INACTIVATION OF SINGLE CHAIN UROKINASE-TYPE PLASMINOGEN ACTIVATOR (PRO-UROKINASE) BY THROMBOMODULIN

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Abstract

The *in vitro* effects of thrombomodulin on the inactivation of single chain urokinase-type plasminogen activator (scu-PA) by thrombin were investigated by incubating scu-PA with varying concentrations of human thrombin, in both the absence and presence of soluble rabbit thrombomodulin. Fifty percent inactivation of scu-PA occurred in 45 minutes at 160 ng/ml thrombin in the absence of thrombomodulin and at 4.6 ng/ml thrombin in the presence of thrombomodulin. No difference was found either in the absence or the presence of thrombomodulin between the inactivation rates of high molecular weight scu-PA, and a low molecular weight scu-PA which lacked the growth factor and kringle domains. Enzyme kinetic experiments with varying concentrations of scu-PA showed that thrombomodulin decreased the K_m of thrombin for scu-PA from 7.8 to 0.43 μM and increased the k_{cat} from 0.30 to 1.2 s^{-1}, corresponding to a 70-fold increase in the second-order rate constant k_{cat}/K_m. SDS-polyacrylamide gel electrophoresis showed that scu-PA was cleaved into two chains upon inactivation by thrombin, and confirmed the acceleration effect of thrombomodulin on inactivation of scu-PA. Thrombomodulin thus not only has anticoagulant properties but is also antifibrinolytic. The acceleration may imply a new mechanism for the regulation of local plasminogen activator activity on the cell surface.

5.1 Introduction

Single chain urokinase-type plasminogen activator (scu-PA)[1], also called pro-urokinase, is the precursor of active two chain urokinase-type plasminogen activator (tcu-PA), a serine protease that activates plasminogen to plasmin (for reviews, see 1-3). Although both forms of urokinase-type plasminogen activator (u-PA) are successfully applied for fibrinolytic therapy, it is not yet known whether u-PA plays an important role in endogenous fibrinolysis. On the other hand, scu-PA probably does play a role in cell migration, tissue destruction and tissue remodelling (for review, see 4). Both scu-PA and tcu-PA have a Mr of about 50,000; tcu-PA consists of two chains with Mr 20,000 and Mr 30,000, held together by a disulphide bridge (5). In certain cell cultures a low molecular weight (LMW) form of scu-PA with Mr 32,000 occurs (6,7) which lacks the N-terminal part (the epidermal growth factor domain and the kringle domain) of the high molecular weight (HMW) form of scu-PA. No significant difference has yet been found between the thrombolytic properties of LMW scu-PA and HMW scu-PA (8,9).

The single chain form of u-PA can be converted to active tcu-PA by hydrolysis of the Lys 158 - Ile 159 peptide bond by plasmin (10), plasma kallikrein (11,12), factor XII (11) and trypsin (13). Thrombin hydrolyses scu-PA at the Arg 156 - Phe 157 bond, leading to an inactive tcu-PA (11,14,15), which is much less sensitive to activation with plasmin (16).

Thrombomodulin is a membrane protein of endothelial cells, which forms a 1:1 complex with thrombin (17) and stimulates the anticoagulant properties of thrombin by accelerating the activation of protein C (18), (for reviews, see 19-22).

The aims of this study were to investigate the effect of thrombomodulin on the inactivation of scu-PA by thrombin and to compare the inactivation by thrombin of HMW and LMW scu-PA. We found that thrombomodulin strongly accelerated the inactivation of both HMW and LMW scu-PA by thrombin.

5.2 Materials and methods

Materials

Both HMW scu-PA and LMW scu-PA, purified from human embryonic kidney cell cultures (23), were donated by Dr J. Henkin of Abbott Laboratories (Abbott Park, Ill). Rabbit lung thrombomodulin and goat anti-rabbit thrombomodulin IgG were purchased from American Diagnostica (New York, NY). Hirudin was from Pentapharm (Basle, Switzerland). Goat non-immune IgG was isolated by two sodium sulphate precipitations (24). Human plasmin and chromogenic substrate, Pyro-Glu-Gly-Arg-pNA (S-2444) were from Kabi Ltd. (Stockholm, Sweden). Aprotinin was from Bayer (Leverkusen, FRG). Human thrombin (T 6759) and bovine serum albumin (BSA, A 7030) were purchased from Sigma (St. Louis, MO). A specific activity of 3.3 NIH U/μg (as indicated by the manufacturer) was used for the calculation of concentrations.

Assay of scu-PA

The activity of scu-PA was determined by measuring the latent amidolytic activity with S-2444 as follows: scu-PA was diluted with 20 mM Tris/HCl, pH 7.5, 100 mM NaCl, 3 mM $CaCl_2$, 0.01% Tween 80, 0.1% BSA, 0.2 ATU/ml hirudin (or more, depending on the amount of thrombin present in the solution) to a concentration of 60-1000 ng/ml and plasmin was added (final concentration 0.1 CU/ml). After 20 minutes activation at 37°C, the amidolytic activity of 75 μl activated scu-PA solution was measured by adding 0.3 mM S-2444 in 175 μl 50 mM Tris/HCl, 38 mM NaCl, 0.01% Tween 80 and 20 KIU/ml aprotinin, pH 8.8. The increase of the optical density at 405 nm was measured during incubation at 37°C by using a Titertek multiskan.

Complexation of thrombin with thrombomodulin

Various concentrations of thrombin up to 0.30 μg/ml (8.1 nM) were incubated with 1.0 μg/ml thrombomodulin (13.5 nM) for 5 minutes at 37°C immediately before each experiment. Functional activity of the thrombomodulin preparation was demonstrated by inhibition of the fibrinogen clotting activity of thrombin by thrombomodulin, tested as described by Esmon et al. (17).

Inactivation of scu-PA

The principle of the assay for scu-PA inactivation has been described previously (15). During 45 minutes at 37°C, 240 ng/ml HMW or LMW scu-PA was incubated with 0-0.6 μg/ml thrombin or with 0-0.3 μg/ml thrombin after pre-incubation with 1 μg/ml thrombomodulin, in 20 mM Tris/HCl, pH 7.5, 100 mM NaCl, 3 mM $CaCl_2$, 0.1% BSA and 0.1% Tween 80. The inactivation was stopped by diluting the mixture in cold hirudin-containing dilution buffer of the assay for scu-PA. In another experiment, varying concentrations of thrombomodulin (0-1 μg/ml) were pre-incubated with 15 ng/ml thrombin before 240 ng/ml HMW scu-PA was added. After 45 minutes at 37°C, the inactivation was stopped and remaining scu-PA was assayed.

Goat anti-rabbit thrombomodulin IgG (40 μg/ml) or goat non-immune IgG (40 μg/ml) was incubated at 37°C for 5 min with 50 ng/ml thrombomodulin. After thrombin was added (15 ng/ml final concentration) and incubated again at 37°C for 5 min, HMW scu-PA (240 ng/ml final concentration) was added and inactivated at 37°C for 45 min. Remaining scu-PA was measured.

The effect of Ca^{2+} on the inactivation of 240 ng/ml HMW scu-PA was studied by adding 0-0.60 μg/ml thrombin alone, or 0-0.12 μg/ml thrombin after incubation with 1 μg/ml thrombomodulin, both in the presence and in the absence of 3 mM $CaCl_2$. After incubation at 37°C for 45 minutes, remaining scu-PA was assayed.

Kinetic experiments

Concentrations of HMW scu-PA ranging from 0.05 to 4 μM were incubated with 4 nM (150 ng/ml) thrombin or 0.04 nM (1.5 ng/ml) thrombin after pre-incubation with 1 μg/ml thrombomodulin in 20 mM Tris/HCl, pH 7.5, 100 mM NaCl, 3 mM $CaCl_2$, 0.1% Tween 80 and 0.1% BSA at 37°C. After 0, 15, 30, 45 and 60 minutes aliquots were taken and the reaction was stopped as described above. Remaining scu-PA was measured with S-2444 after activation with plasmin and the initial rates of scu-PA inactivation were determined from the linear parts of the time curves.

SDS-polyacrylamide gel electrophoresis

SDS-PAGE was performed according to the method of Laemmli (25), using a 12% polyacrylamide gel. Samples of 30 μl were diluted 1:1 in sample buffer containing 4% (w/v) SDS, 20% (w/v) glycerol, 0.005% (w/v) bromophenol blue and 4% (w/v) 2-mercaptoethanol in 125 mM Tris/HCl, pH 6.8 and incubated for 5 min at 100°C. A calibration kit with standard proteins was obtained from Pharmacia LKB Biotechnology (Uppsala, Sweden).

5.3 Results

Inactivation of HMW and LMW scu-PA

In order to study the effect of thrombomodulin on the inactivation of scu-PA by thrombin, 240 ng/ml HMW and LMW scu-PA were incubated for 45 minutes at 37°C with varying amounts of thrombin, both in the absence and presence of 1 μg/ml thrombomodulin. Remaining scu-PA, not inactivated by thrombin, was activated with plasmin and quantified using S-2444. Fifty percent of scu-PA was inactivated by 160 ± 41 ng/ml (mean ± SD, n = 5) thrombin in the absence of thrombomodulin or by only 4.6 ± 1.4 (n = 4) ng/ml thrombin in the presence of thrombomodulin (Fig. 1). No difference was found in the relative inactivation rate between LMW scu-PA and HMW scu-PA, either in the absence or in the presence of thrombomodulin.

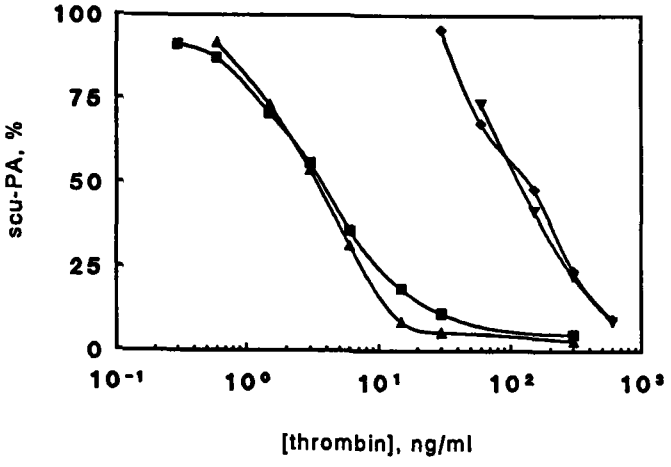

Figure 1. Effect of thrombomodulin on the inactivation of HMW scu-PA (■ and ◆) and LMW scu-PA (▲ and ▼) by thrombin. The percentages of scu-PA remaining after incubation for 45 min at 37°C with varying concentrations of thrombin in the absence (◆,▼) or in the presence of 1 μg/ml thrombomodulin (■,▲) were quantified with S-2444 after activation with plasmin. The 100% values were not affected by either the absence or presence of thrombomodulin.

Figure 2 shows that remaining scu-PA gradually decreased at increasing thrombomodulin concentrations and a fixed thrombin concentration of 15 ng/ml. In

order to calculate the Kd for the thrombin-thrombomodulin complex from these data, the percentages of remaining scu-PA were converted into concentrations of the thrombin-thrombomodulin complex, by reference to the standard curve prepared with thrombin in the presence of 1 μg/ml thrombomodulin (Fig. 1) and by assuming that all thrombin had formed a 1:1 complex at 1 μg/ml thrombomodulin. The concentrations of free and bound thrombomodulin were calculated and plotted double-reciprocally (Fig. 2, inset), resulting in an apparent Kd of 0.57 nM for the thrombin-thrombomodulin complex.

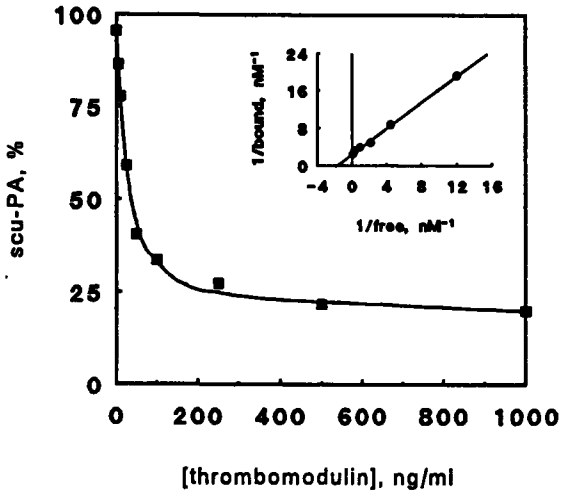

Figure 2. Effect of varying concentrations of thrombomodulin on the inactivation of HMW scu-PA by thrombin. Thrombin (15 ng/ml) was pre-incubated with 0-1 μg/ml thrombomodulin for 5 min at 37°C and then incubated with 240 ng/ml HMW scu-PA for 45 min at 37°C. The amounts of free and bound thrombomodulin were calculated from the activities of the thrombin-thrombomodulin mixtures (see text) and plotted double-reciprocally to determine the Kd (inset).

The neutralizing effect of goat antibodies against thrombomodulin was studied in Fig. 3. About 4% scu-PA was inactivated by 15 ng/ml thrombin alone, while 56% was inactivated by the same concentration thrombin complexed with 50 ng/ml thrombomodulin. The antibodies almost completely quenched the accelerating effect of thrombomodulin on the inactivation of HMW scu-PA, whereas goat non-immune IgG had only a minor effect.

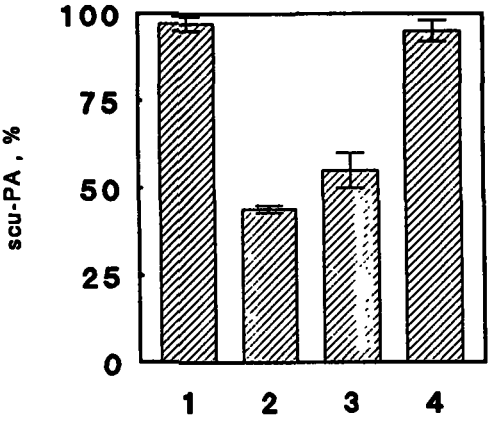

Figure 3. Quenching of the accelerating effect of thrombomodulin by specific antibodies. HMW scu-PA (240 ng/ml) was incubated for 45 min at 37°C with 15 ng/ml thrombin (1), with 15 ng/ml thrombin preincubated in 50 ng/ml thrombomodulin (2), with 15 ng/ml thrombin, 50 ng/ml thrombomodulin, and 40 μg/ml goat non-immune IgG (3) or 40 μg/ml goat anti-thrombomodulin IgG (4). Standard deviations are presented as horizontal lines (n = 3).

SDS-PAGE of inactivated HMW and LMW scu-PA

HMW and LMW scu-PA were incubated with a low concentration of thrombin (75 ng/ml) both with and without 1 μg/ml thrombomodulin or with a high concentration of thrombin (1.2 μg/ml) and subjected to SDS-PAGE (Fig. 4). The low concentration of thrombin partially cleaved HMW scu-PA in two subunits: one of Mr 32,000 and one of Mr 20,000 or 22,000 (lane 3). In contrast, the thrombin-thrombomodulin complex completely cleaved HMW scu-PA (lane 4), as did the high concentration of thrombin (lane 5). Comparison of the extent of cleavage in lane 3 and 4 clearly shows the accelerating effect of thrombomodulin. As expected, inactivation of LMW scu-PA did not lead to a significant shift of the protein band, as a peptide of only 13 amino acids was supposed to be cut off.

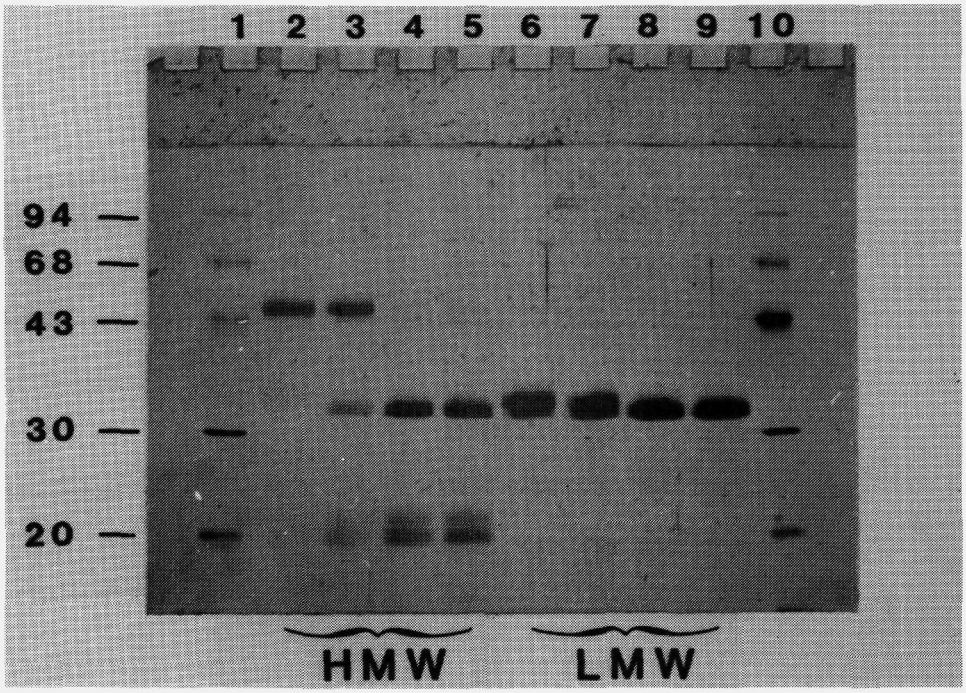

Figure 4. SDS-PAGE of inactivated HMW and LMW scu-PA. HMW and LMW scu-PA (62.5 μg/ml) were incubated for 45 min at 37°C in 20 mM Tris/HCl, 100 M NaCl, 3 mM $CaCl_2$, and 0.1% Tween 80, pH 7.5, as specified below. Lanes 1 and 10: Standard proteins with Mr 94,000, Mr 68,000, Mr 43,000, Mr 30,000, Mr 20,000. Lane 2: HMW scu-PA; Lane 3: HMW scu-PA after inactivation with thrombin (75 ng/ml); Lane 4: HMW scu-PA after inactivation with thrombin (75 ng/ml) in the presence of 1.0 μg/ml thrombomodulin; Lane 5: HMW scu-PA after inactivation with a high concentration of thrombin (1.2 μg/ml); Lane 6-9: as lanes 2-5 except that HMW scu-PA was replaced by LMW scu-PA. All samples were reduced with 2-mercaptoethanol. Protein bands were visualized with silver staining (26).

Kinetics of the inactivation of HMW scu-PA

Varying amounts of HMW scu-PA were incubated with thrombin or with thrombin after preincubation with excess thrombomodulin. Kinetic analysis shows that the inactivation rate of scu-PA by both thrombin and the thrombin-thrombomodulin complex obeyed Michaelis-Menten kinetics. Using Lineweaver-Burk plots (Fig. 5), the K_m and k_{cat} were determined to be 7.8 μM and 0.30 s^{-1}, respectively, in the case of thrombin and 0.43 μM and 1.2 s^{-1}, respectively, for the thrombin-thrombomodulin complex (Table 1). The second-order rate constant (k_{cat}/K_m) of thrombin increased from 0.039 to 2.7 $\mu M^{-1}.s^{-1}$ (70 times) by complex formation with thrombomodulin.

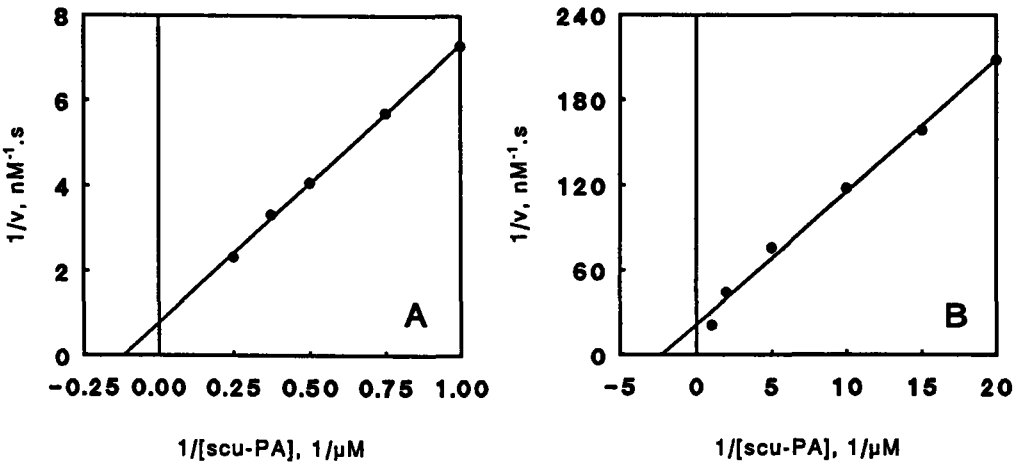

Figure 5. Lineweaver-Burk plots of the inactivation of HMW scu-PA. At varying concentrations of scu-PA, the inactivation rate of thrombin (A) and thrombin in the presence of thrombomodulin (B) was measured as described under Materials and Methods. The k_{cat} and K_m values were determined by using the least squares method and are summarized in Table 1.

Table 1. Kinetic parameters of the inactivation of HMW scu-PA by thrombin and thrombin-thrombomodulin complex (derived from Fig. 5) and a comparison with literature data for the activation of protein C.

Enzyme	Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)
Thrombin	scu-PA	7.8	0.30	0.039
Thrombin + thrombomodulin	scu-PA	0.43	1.2	2.7
Thrombin*	protein C	60	0.02	0.00033
Thrombin + thrombomodulin‡	protein C	7.6	6.2	0.81

* According to ref. 27; ‡ according to ref. 28.

Effect of Ca^{2+} on the inactivation of HMW scu-PA

In the presence of Ca^{2+} more thrombin was required to inactivate HMW scu-PA than in the absence of Ca^{2+}. This was observed both in the presence (2.3 times more thrombin to achieve 50% inactivation) and in the absence (9 times more) of thrombomodulin (Fig.

6). An experiment with varying Ca^{2+} concentrations (0-6 mM) revealed that under both conditions a plateau was reached at 3 mM Ca^{2+} (not shown).

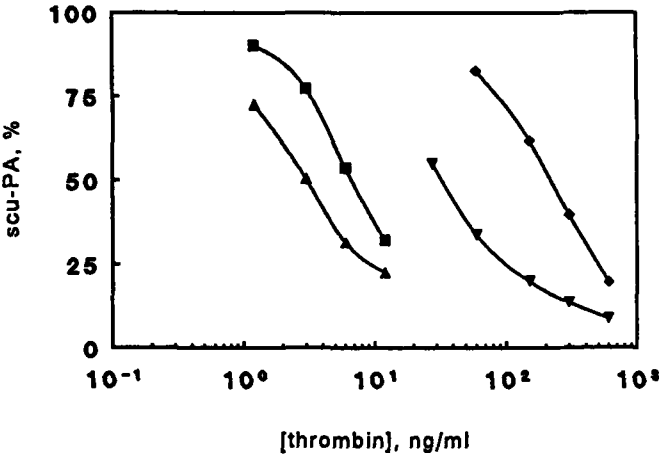

Figure 6. Effect of Ca^{2+} on the inactivation of HMW scu-PA. Remaining scu-PA was determined after inactivation by thrombin in the presence of 3 mM Ca^{2+} (◆) or absence of Ca^{2+} (▾) and by thrombin-thrombomodulin complex in the presence of 3 mM Ca^{2+} (■) or absence of Ca^{2+} (▲). The concentration of scu-PA was 240 ng/ml, the concentration of thrombomodulin was 1 μg/ml; for experimental details see Materials and Methods.

5.4 Discussion

This study shows an accelerating effect of solubilized rabbit thrombomodulin on the inactivation of scu-PA by human thrombin. This finding was supported by the following observations: (1) The effect of thrombomodulin was dose-dependent and corresponded with an apparent Kd for the thrombin-thrombomodulin complex of 0.57 nM, which is very similar to previously reported values of 0.48 nM and 0.54 nM (18,29). (2) Goat anti-rabbit thrombomodulin IgG inhibited the accelerating effect of thrombomodulin. (3) The action of thrombomodulin was accompanied with the conversion of scu-PA into a tcu-PA which was indistinguishable on SDS-PAGE from the tcu-PA formed by a high concentration of thrombin.

The inactivation of HMW scu-PA by the thrombin-thrombomodulin complex as well as by thrombin alone obeyed Michaelis-Menten kinetics. The k_{cat}/K_m for thrombin alone was 0.039 $\mu M^{-1}.s^{-1}$ and increased 70-fold to 2.7 $\mu M^{-1}.s^{-1}$ for the thrombin-thrombomodulin complex. It is difficult to establish whether the latter rate constant is high enough to be significant *in vivo*. However, the rate constant is of the same order of magnitude as that for the activation of scu-PA by plasmin (30,31). In addition, the k_{cat}/K_m for the activation of protein C by the thrombin-thrombomodulin complex in an assay system as employed in this study, is 0.81 $\mu M^{-1}.s^{-1}$ (27), which is also of the same order of magnitude (Table 1). The activation rate of protein C by the complex further increases on cell surfaces and in the presence of phospholipids (18,28). It still has to be determined whether the

inactivation rate of scu-PA on cell surfaces is also higher than in the solubilized system.

In the absence of endothelial cells Ca^{2+} reduces the rate of protein C activation by thrombin on the one hand and stimulates the activation rate by the thrombin-thrombomodulin complex on the other (32). The thrombin-thrombomodulin interaction is Ca^{2+}-independent (17). In contrast to the diverging effects of Ca^{2+} on protein C activation, Ca^{2+} reduced the inactivation of scu-PA by both thrombin and thrombin-thrombomodulin complex. It is not yet clear whether the active site of thrombin is affected or whether the substrate scu-PA is changed by a hitherto unknown Ca^{2+}-binding phenomenon.

The two molecular weight forms (HMW and LMW) of scu-PA were equally sensitive to inactivation by thrombin or the thrombin-thrombomodulin complex. LMW scu-PA, a potential thrombolytic agent, has, therefore, no advantage or disadvantage compared with HMW scu-PA in terms of sensitivity for inactivation in thrombolytic therapy.

The physiological importance of the acceleration of scu-PA inactivation by thrombomodulin may lie in the close regulation of local u-PA activity on surfaces of endothelial cells and other cells which express thrombomodulin. Through this mechanism thrombin and thrombomodulin may affect processes such as cell migration, tissue destruction and remodelling. In this respect, it is interesting to note that fetomodulin, a surface marker protein of fetal development, has recently been demonstrated to be identical to thrombomodulin (33). During embryonic development this marker is not only expressed in vasculatures but also in nonvascular tissues. Consequently, it has been suggested that thrombomodulin has another function, in addition to that of an anticoagulant (33). This study indicates such a function, implying the regulation of u-PA activity during embryonic development.

If scu-PA plays a role in endogenous fibrinolysis, thrombomodulin should be considered as a protein with anti-fibrinolytic properties in addition to its well-known anticoagulant properties. Both properties may be necessary, either simultaneously or successively, to regulate size and longevity of fibrin deposits. Inactivation of scu-PA by thrombomodulin-bound thrombin may also occur during thrombolytic therapy with scu-PA, the more so as thrombin seems to be formed in patients during thrombolysis (34). This would be an additional argument for administration of thrombin inhibitors simultaneously with the thrombolytic agent scu-PA (35).

5.6 Acknowledgements

This study was supported by the Netherlands Heart Foundation (Grant 87.041 and Established Investigatorship of D.C.R.). We thank Drs. J. Henkin and A.A. Sasahara, Abbott Laboratories for generously providing HMW and LMW scu-PA.

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Appendix to CHAPTER 5

INACTIVATION OF HIGH AND LOW MOLECULAR WEIGHT SINGLE-CHAIN UROKINASE-TYPE PLASMINOGEN ACTIVATOR (PRO-UROKINASE) BY THROMBIN IN THE PRESENCE OF THROMBOMODULIN

Letter to the Editor

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Thrombosis and Haemostasis 1993; 67: 88.

Dear Sir,

Two independent studies have recently shown that the inactivation of single-chain urokinase-type plasminogen activator (scu-PA) by thrombin is accelerated by thrombomodulin (1,2). The two studies disagree, however, about the role of the amino-terminal part of the scu-PA molecule in the inactivation reaction. De Munk et al. (1) have stated that "no difference was found in either the absence or the presence of thrombomodulin between the inactivation rates of high molecular weight (HMW) scu-PA, and a low molecular weight (LMW) scu-PA which lacked the growth factor and kringle domains". In contrast, by studying a Delta 125 rscu-PA mutant which lacks amino acid residue 11-135, Molinari et al. (2) have postulated that "the amino-terminal sequence of rscu-PA, containing the epidermal growth factor-like and the kringle domains is involved in the cofactor effect of thrombomodulin on scu-PA inactivation by thrombin". In the present study we have tested whether the apparent discrepancy should be ascribed either to differences in methodology or to differences in scu-PA preparations. To this end, the scu-PA preparations of the two research groups were tested side-by-side in one laboratory.

The scu-PA preparations (240 ng/ml) were incubated with various concentrations of thrombin for 45 min at 37°C, and remaining scu-PA was assayed (1). Table 1 summarizes the thrombin concentrations required for 50% scu-PA inactivation both in the absence and in the presence of an excess of thrombomodulin (1 μg/ml). The two scu-PA preparations of De Munk et al. (1), obtained from a human kidney cell culture at Abbott Laboratories (Abbott Park, IL), were inactivated for 50% at 160 ± 41 and 4.6 ± 1.4 ng/ml thrombin in the absence and presence of thrombomodulin, respectively. Recombinant scu-PA (rscu-PA) and its LMW mutant, expressed in Chinese hamster ovary cells at the University of Leuven, Belgium (3) were tested as control preparations. They appeared to be as sensitive to thrombin and the thrombin-thrombomodulin complex, respectively, as were scu-PA and LMW scu-PA obtained from Abbott Laboratories. The recombinant scu-PA of Molinari et al. (2), prepared by expression in E.coli at Farmitalia (Milano, Italy), also behaved similarly. In contrast, the Delta 125 rscu-PA mutant of Molinari et al., lacking the growth factor and kringle domains, required somewhat more thrombin (271 ng/ml) than scu-PA or rscu-PA in the absence of thrombomodulin, and needed significantly more thrombin (49 ng/ml) than scu-PA or rscu-PA in the presence of thrombomodulin. Thrombomodulin thus accelerated the inactivation of Delta 125 rscu-PA by thrombin only moderately (5.5 fold as compared to 35-72 fold for the other scu-PA preparations). These results show that the apparent discrepancy between De Munk et al. (1) and Molinari et al. (2) cannot be ascribed to differences in methodology, as their results were confirmed in a side-by-side comparison of the scu-PA preparations. It is

concluded that the inactivation of scu-PA and scu-PA mutants by the thrombin-thrombomodulin complex does not require the presence of the growth factor and kringle domains in the scu-PA molecule. The relatively slow inactivation of the Delta 125 rscu-PA mutant may be related to its amino-terminus, which is 18 amino acid residues longer than the amino-terminus of LMW scu-PA and LMW rscu-PA.

Table 1. Inactivation of various forms of scu-PA by thrombin, in the absence and in the presence of thrombomodulin (TM). The data indicate the thrombin concentrations, which were required for 50% inactivation.

scu-PA Form	Source	Thrombin concentration (ng/ml) Without TM	With TM
scu-PA	Abbott	160 ± 41*	4.6 ± 1.4*
LMW scu-PA	Abbott	160 ± 41*	4.6 ± 1.4*
rscu-PA	Leuven	209	2.9
LMW rscu-PA	Leuven	176	3.1
rscu-PA	Farmitalia	214	5.3
Delta 125 rscu-PA	Farmitalia	271	49

* Values represent mean ± SD and have been taken from ref. 1. The other values represent the mean of two determinations (the difference between duplicate determinations was 15% or less).

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This study was supported by the Netherlands Heart Foundation (grant 87.041 and Established Investigatorship of D.C.R.).

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CHAPTER 6

ROLE OF THE GLYCOSAMINOGLYCAN COMPONENT OF THROMBOMODULIN IN ITS ACCELERATION OF THE INACTIVATION OF SINGLE-CHAIN UROKINASE-TYPE PLASMINOGEN ACTIVATOR BY THROMBIN

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Synopsis

Thrombomodulin (TM), a membrane proteoglycan on endothelial cells, binds thrombin in a 1:1 complex, accelerates the protein C activation by thrombin, promotes the thrombin inactivation by antithrombin III and inhibits the procoagulant properties of thrombin. The inactivation of single-chain urokinase-type plasminogen activator (scu-PA) by thrombin is accelerated about 70-fold by TM [De Munk, Groeneveld & Rijken (1991) J. Clin. Invest. 88, 1680-1684]. The present study investigates the role of the O-linked glycosaminoglycan moiety of TM in the latter reaction. In the presence of an excess of a fully-glycosylated soluble recombinant human TM mutant (high-Mr rec-TM), 0.11 nM thrombin inactivated 50% of 4.4 nM scu-PA in 45 min at 37°C. In the presence of a soluble recombinant TM mutant lacking the glycosaminoglycans (low-Mr rec-TM), 1.9 nM thrombin was needed to inactivate 50% scu-PA, as compared with 4.7 nM thrombin in the absence of TM. Using the scu-PA inactivation assay the dissociation constant for the thrombin-TM interaction was found to be 0.4 nM for high-Mr rec-TM and 14 nM for low-Mr rec-TM. Treatment of high-Mr rec-TM with chondroitinase ABC to digest the glycosaminoglycans decreased the accelerating effect to the level of low-Mr rec-TM. A similar decrease was observed after treatment of solubilized rabbit TM with chondroitinase ABC. As expected, chondroitinase ABC had no influence on the acclerating effect of low-Mr rec-TM. The free glycosaminoglycans obtained by alkaline treatment of TM or chondroitin sulphate A also accelerated the inactivation of scu-PA by thrombin, but about 1000-fold higher concentrations than with TM were needed to obtain the same acceleration. It is concluded that the major glycosaminoglycan of TM plays a pivotal role in the inactivation of scu-PA by the TM-thrombin complex, both in the formation and in the activity of the complex.

6.1 Introduction

The serine protease single-chain urokinase-type plasminogen activator (scu-PA), also called pro-urokinase, can, when converted to two-chain urokinase-type plasminogen activator (tcu-PA), activate plasminogen to plasmin. The activator probably has an important role in fibrinolysis and in the maintenance of the haemostatic balance (for reviews see Lijnen *et al.*, 1987*a*; Gurewich, 1988, de Munk & Rijken, 1990). In contrast to plasmin and kallikrein which cleave scu-PA between Lys 158 and Ile 159 resulting in an active two-chain molecule, thrombin cleaves scu-PA between Arg 156 and Phe 157, resulting in an

inactive two-chain molecule (Bernik & Oller, 1977; Ichinose *et al.*, 1986; Gurewich & Pannell, 1987). Thrombin-inactivated scu-PA is very poorly activated by plasmin (Lijnen *et al.*, 1987*b*).

Thrombomodulin (TM), an endothelial cell membrane proteoglycan, binds thrombin with high affinity in a 1:1 complex. The protein stimulates the anticoagulant properties of thrombin by accelerating the activation of protein C and inhibits the procoagulant properties of thrombin (for reviews, see Freyssinet & Cazenave, 1988; Esmon, 1987; Esmon, 1989; Dittman & Majerus, 1990). Beside these anticoagulant effects, TM may also have an antifibrinolytic effect by accelerating the inactivation of scu-PA by thrombin (De Munk *et al.*, 1991; Molinari *et al.*, 1992).

TM contains many N- and O-linked glycans (Bourin *et al.*, 1986; 1990*b*; Koyama *et al.*, 1991*a*; Parkinson *et al.*, 1992). One O-linked chondroitin sulphate-like glycosaminoglycan (CSGAG) in TM constitutes a secondary binding site for thrombin (Bourin *et al.*, 1988; Preissner *et al.*, 1990) in addition to the primary binding site on the epidermal growth factor domains 5 and 6 of TM (Kurosowa *et al.*, 1988; Hayashi *et al.*, 1990). This CSGAG potentiates inhibition of thrombin's activity towards fibrinogen and other bulky substrates (Bourin *et al.*, 1988; Preissner *et al.*, 1990; Bourin & Lindahl, 1990; Parkinson *et al.*, 1990; Nawa *et al.*, 1990), and is essential for the acceleration of thrombin inhibition by antithrombin III (Preisner *et al.*, 1990; Bourin *et al.*, 1988; Bourin & Lindahl, 1990; Nawa *et al.*, 1990; Koyama *et al.*, 1991*b*). The CSGAG also significantly enhances protein C activation by the thrombin-TM complex at physiological calcium concentrations (Parkinson *et al.*, 1990; Parkinson *et al.*, 1992). At low calcium concentrations, the CSGAG has, however, a less critical function in the activation of protein C (Preissner *et al.*, 1990; Bourin & Lindahl, 1990; Parkinson *et al.*, 1990, 1992).

Various molecular models have recently been designed to illustrate the role of the CSGAG in the interaction between TM, thrombin and different substrates (Bourin & Lindahl, 1990; Preissner *et al.*, 1990; Koyama *et al.*, 1991*b*). The aim of this study was to investigate the role of the O-linked chondroitin sulphate-like glycosaminoglycan of TM in the acceleration of the inactivation of scu-PA by thrombin. For that purpose we used two glycoforms of a soluble human recombinant thrombomodulin (rec-TM), a high-Mr rec-TM and a low-Mr rec-TM lacking the O-linked CSGAG (Parkinson *et al.*, 1990, 1992). In addition we modified TM chemically and enzymatically.

6.2 Materials and methods

Materials

Human scu-PA and low molecular weight scu-PA, lacking the NH_2-terminal part of the

molecule (the epidermal growth factor domain and kringle domain), were isolated from embryonic kidney cell cultures (Badylak *et al.*, 1988) and donated by Dr. J. Henkin, Abbott Laboratories (Abbott Park, IL, USA). Rabbit lung TM (Mr 74,000; Esmon *et al.*, 1982) was purchased from American Diagnostica Inc. (Greenwich, CT, USA). Soluble human high- and low-Mr recombinant thrombomodulin (rec-TM) with Mr 105,000 and Mr 75,000 respectively, were purified from conditioned medium of stable 293 cell transformants, as recently described (Parkinson *et al.*, 1990, 1992). Hirudin was from Pentapharm (Basel, Switzerland). Human plasmin and the chromogenic substrate Pyro-Glu-Gly-Arg-pNA (S-2444) were from Kabi AB (Stockholm, Sweden). Aprotinin was from Bayer AG (Leverkusen-Bayerwerk, Germany). Human thrombin (T6759), bovine serum albumin (BSA) (A7030), chondroitinase ABC (C2905), pepstatin (P4265), leupeptin (L2884), chondroitin sulphate A (C8529) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). A specific activity of 3.3 NIH U/μg for thrombin (as indicated by the manufacturer) and Mr 37,000 were used for the calculation of concentrations.

Assay of scu-PA

The amount of scu-PA was determined by activating scu-PA (3.7 nM) in 20 mM Tris/HCl, pH 7.5, 100 mM NaCl, 3 mM $CaCl_2$, 0.1% BSA, 0.1% Tween-80 and hirudin (see below), with plasmin (final concentration 0.1 CU/ml or 45 nM) for 20 min at 37°C. From the activated scu-PA solution, 75 μl was added to 175 μl 0.43 mM S-2444 in 50 mM Tris/HCl, 38 mM NaCl, 0.01% Tween-80 and 20 KIU/ml aprotinin, pH 8.8. The increase of the optical density at 405 nm was measured during incubation at 37°C by using a Titertek multiskan (Eflab Oy, Finland). In this assay scu-PA was thus fully activated before measurement of tcu-PA.

Inactivation of scu-PA

The assay for inactivation of scu-PA was performed by incubating 4.4 nM scu-PA with 0-16 nM thrombin in the absence or presence of rabbit TM, TM mutants CSGAG or chondroitin sulphate A in 20 mM Tris/HCl, pH 7.5, 100 mM NaCl, 3 mM $CaCl_2$, 0.1% BSA, 0.1% Tween-80, for 45 min at 37°C. Thrombin was preincubated with the components in the incubation buffer for 5 min at 37°C, immediately before adding scu-PA. The reaction was stopped by adding hirudin (with a final concentration of 0.2 U/ml or more, depending on the amount of thrombin present in the solution) and scu-PA activity was determined.

Chondroitinase ABC treatment

Rabbit TM (81 nM, 500 μl) and low- and high-Mr rec-TM (720 nM, 200 μl) were

incubated overnight at 37°C with and without chondroitinase ABC in 50 mM Tris/HCl, pH 8.0, 100 mM NaCl, 30 μM sodium acetate, in the presence of 2.5 mM 1,10-phenanthroline, 10 μg/ml pepstatin, and 20 μg/ml leupeptin (Parkinson *et al.*, 1990). The chondroitinase ABC concentration amounted to 250 mU/ml and was enhanced to 500 mU/ml after 6 hrs.

Alkaline β-elimination

High-Mr rec-TM (1.15 μM, 15 μl) was added to 135 μl 0.1 M NaCl, 0.56 M NaOH and 0.1% Tween-80 and incubated for 15 h at 4°C to release O-linked saccharides from the protein (Bourin *et al.*, 1990). The solution was neutralized with HCl and tested in the inactivation assay of scu-PA. It is assumed that the CSGAG accounts for 10% by weight of the high-Mr rec-TM (Bourin *et al.*, 1990).

6.3 Results

To compare the accelerating effect of different TM preparations on the thrombin inactivation of scu-PA, various concentrations of thrombin were incubated with 4.4 nM scu-PA for 45 min at 37°C, in the absence or presence of excess rabbit TM (13.5 nM), human high-Mr rec-TM (13.5 nM) or human low-Mr rec-TM (27 nM). The residual native scu-PA was activated by plasmin and quantified using S-2444 (Fig. 1). Fifty percent scu-PA

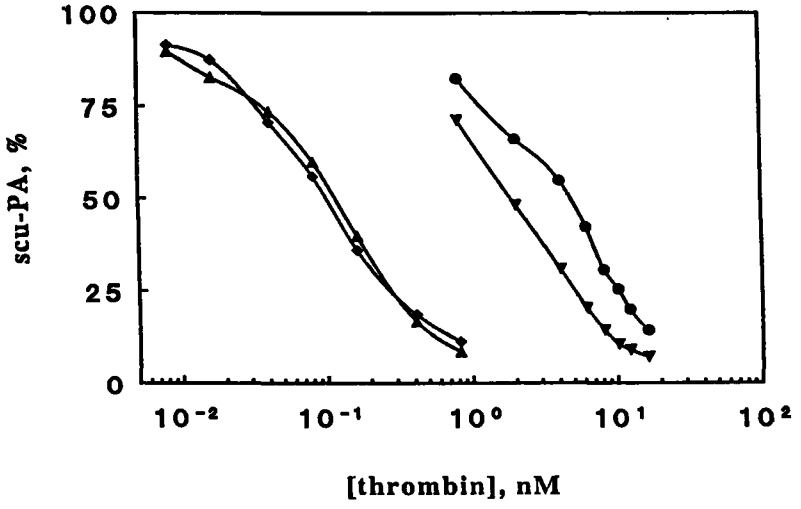

Figure 1. Effect of different types of TM on the inactivation of scu-PA by thrombin. The percentages of scu-PA remaining after incubation for 45 min at 37°C with varying concentrations of thrombin in the absence (•) or in the presence of excess rabbit TM (13.5 nM) (◆), high-Mr rec-TM (13.5 nM) (▲) or low-Mr rec-TM (27 nM) (▼) were quantified with S-2444 after activation with plasmin.

Table 1. Inactivation of low molecular weight scu-PA. Data in the table represent the concentrations of thrombin (nM), inactivating 50% scu-PA in the absence or presence of different types of thrombomodulin (TM). The values for high molecular weight scu-PA were taken from Fig. 1. For experimental details see text.

TM	thrombin concentration (nM)	
	low molecular weight scu-PA	high molecular weight scu-PA
Rabbit TM	0.09	0.10
high-Mr rec-TM	0.13	0.11
low-Mr rec-TM	1.8	1.9
none	5.0	4.7

was inactivated by 0.10 nM thrombin in complex with rabbit TM, by 0.11 nM thrombin in complex with high-Mr rec-TM, by 1.9 nM thrombin in complex with low-Mr rec-TM and by 4.7 nM thrombin in the absence of TM. All three thrombin-TM complexes as well as free thrombin inactivated low molecular weight scu-PA (7.3 nM) to the same extent as the intact scu-PA (Table 1). This indicated that the epidermal growth factor domain and the kringle domain of scu-PA are not involved in the inactivation reaction.

With increasing concentrations of high- and low-Mr rec-TM and a fixed thrombin concentration the acceleration of the scu-PA inactivation reached a maximum level (Fig. 2), at which all the thrombin was assumed to have formed a 1:1 complex with rec-TM. The apparent Kd for this complex was determined as follows (De Munk *et al.*, 1991). The percentages of remaining scu-PA were converted into concentrations of thrombin-rec-TM complex, by reference to the standard curves prepared with various concentrations of thrombin in the presence of an excess of the corresponding rec-TM (Fig. 1). The concentrations of free and bound rec-TM were then calculated and double reciprocal plots (Figs. 2A and B, inset) were used to determine the Kd values of the complexes. The apparent Kd for the thrombin-low-Mr rec-TM complex was 14 $\pm$ 5 nM and 0.4 $\pm$ 0.1 nM (n = 3) for the thrombin-high-Mr rec-TM complex.

After treatment with chondroitinase ABC which destroys the CSGAG moiety, rabbit TM (13.5 nM), high-Mr rec-TM (13.5 nM), and low-Mr rec-TM (27 nM) were incubated with varying concentrations of thrombin and 4.4 nM scu-PA for 45 min at 37°C. Remaining scu-PA was determined. Figure 3 shows that the activities of high-Mr rec-TM and rabbit TM were markedly reduced and dropped almost to the activity of low-Mr rec-TM. By contrast, the activity of low-Mr rec-TM was unaffected by chondroitinase ABC (Fig. 3). Rabbit TM incubated without chondroitinase ABC as a control showed no significant loss of activity (not shown).

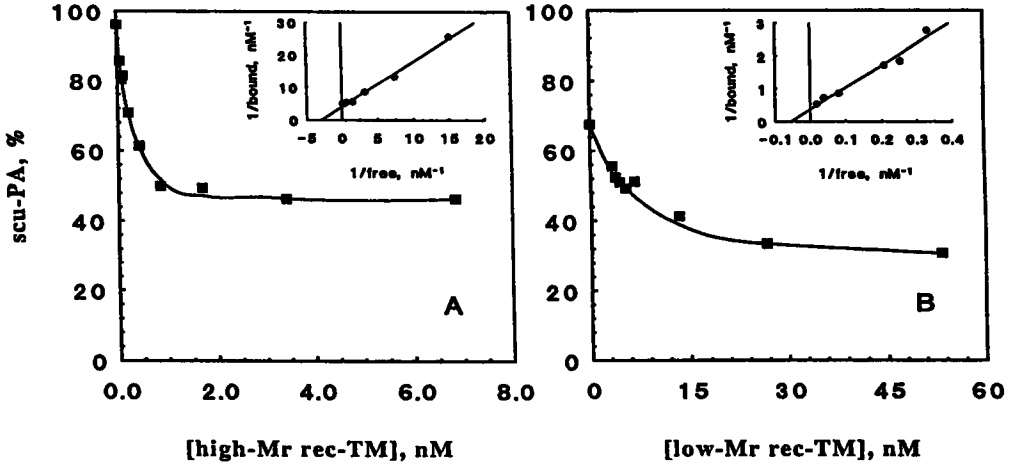

Figure 2. Effect of varying concentrations of the rec-TM glycoforms on the inactivation of scu-PA by thrombin. Thrombin was incubated at a concentration of 0.2 nM with 0-6.7 nM high-Mr rec-TM (A) or at a concentration of 2 nM with 0-54 nM low-Mr rec-TM (B) and 4.4 nM scu-PA for 45 min at 37°C. Remaining scu-PA was determined. The amounts of free and bound TM were calculated (see text) and plotted reciprocally to determine the Kd values (insets).

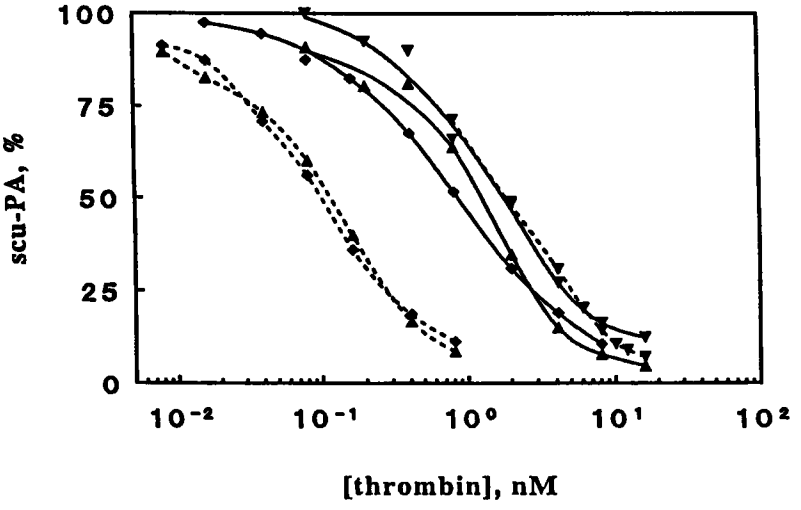

Figure 3. Chondroitinase ABC treatment of rec-TM and rabbit TM. Scu-PA and thrombin were incubated with chondroitinase ABC-treated rabbit TM (◆), high-Mr rec-TM (▲), low-Mr rec-TM (▼). The dashed lines represent curves obtained with the corresponding untreated TM preparations, and were redrawn from Fig. 1. Remaining scu-PA was determined.

The O-linked CSGAG was hydrolysed from the peptide chain of high-Mr rec-TM by alkaline β-elimination. The peptide chain was destroyed as was shown by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Various concentrations of high-Mr rec-TM-derived CSGAG (0-2 μg/ml), chondroitin sulphate A (0-100 μg/ml), which is closely related to the CSGAG moiety on TM (Bourin *et al.*, 1990*b*; Nawa *et al.*, 1990) or intact high-Mr rec-TM (0-6.7 nM or 0-700 ng/ml, corresponding to about 0-70 ng/ml CSGAG) were incubated with 2 nM thrombin and 4.4 nM scu-PA for 45 min at 37°C. Remaining scu-PA was measured (Fig. 4). Both chondroitin sulphate A and the free CSGAG from TM showed a significant and roughly equal acceleration of the scu-PA inactivation by thrombin. However, high concentrations were needed, about 1000-fold higher than with intact high-Mr rec-TM.

Reconstitution experiments were performed by incubating scu-PA (4.4 nM) and thrombin for 45 min at 37°C in the presence of both low-Mr rec-TM (27 nM) and CSGAG (0.14 μg/ml or 13.5 nM), isolated from high-Mr rec-TM. Figure 5 shows that the accelerating effects of the two compounds were additive, but restoration to the level of intact high-Mr rec-TM was not achieved. These results indicate that the polypeptide of rec-TM and the CSGAG must be covalently linked for expression of full activity.

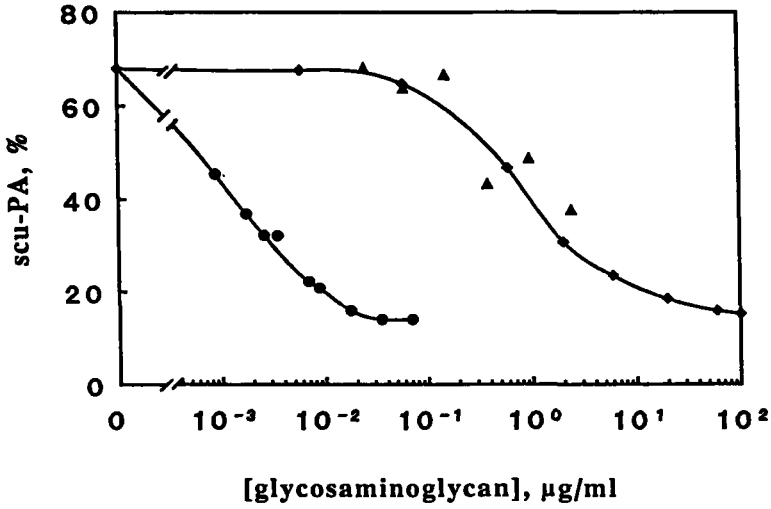

Figure 4. Inactivation of scu-PA by thrombin accelerated by different CSGAG preparations. Scu-PA was incubated for 45 min at 37°C with 2 nM thrombin in the presence of varying concentrations of intact high-Mr rec-TM (assuming 10% of the molecular weight is CSGAG) (•), CSGAG obtained from high-Mr rec-TM by alkaline β-elimination (▲) and chondroitin sulphate A (◆). Remaining scu-PA was determined.

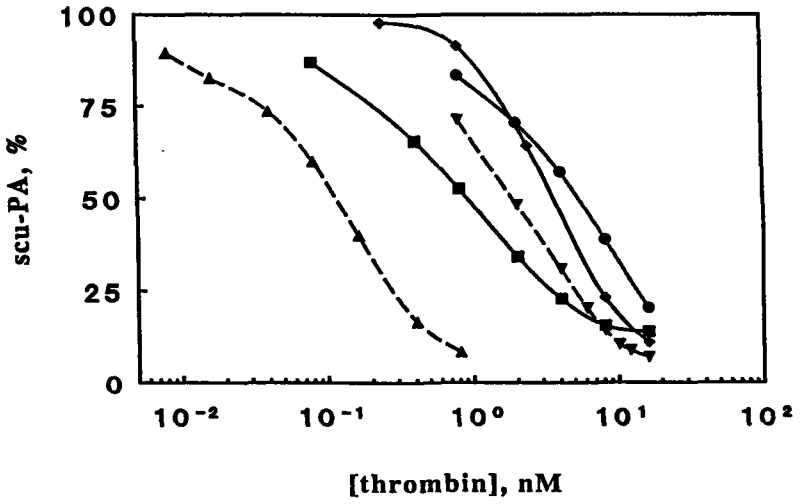

Figure 5. Reconstitution of low-Mr rec-TM with CSGAG from high-Mr rec-TM. Scu-PA and thrombin were incubated alone (•), with CSGAG (0.14 μg/ml or 13.5 nM) obtained by alkaline β-elimination of high-Mr rec-TM (◆) and in the presence of both low-Mr rec-TM (27 nM) and CSGAG (0.14 μg/ml or 13.5 nM) from high-Mr rec-TM (■). Remaining scu-PA was determined. Dotted lines represent inactivation of scu-PA by thrombin in the presence of low-Mr rec-TM (27 nM) (▾) and high-Mr rec-TM (13.5 nM) (▴), taken from Fig. 1.

6.4 Discussion

Recent studies have shown that the anticoagulant protein TM may also have an antifibrinolytic activity by accelerating the inactivation of scu-PA by thrombin (De Munk *et al.*, 1991; Molinari *et al.*, 1992). The present study shows that this property of TM strongly depends on the presence of an O-linked CSGAG, as demonstrated with two glycoforms of human soluble rec-TM as well as with rabbit TM.

Fifty percent scu-PA was inactivated in 45 min with 4.7 nM free thrombin and only 0.10 or 0.11 nM thrombin respectively in the presence of excess rabbit TM or human high-Mr rec-TM, indicating that the two TM preparations had equal accelerating effects. In the presence of an excess human low-Mr rec-TM, lacking the O-linked CSGAG (Parkinson *et al.*, 1990, 1992; Koyama *et al.*, 1991*a*), 1.9 nM thrombin was needed to inactivate fifty percent scu-PA, thus showing only a slight accelerating effect.

The conclusion that only the CSGAG is responsible for the difference in activity of high-Mr and low-Mr rec-TM was supported by the experiments in which treatment of high-Mr rec-TM with chondroitinase ABC led to the level of activity of the low-Mr form. The free CSGAG component, obtained from high-Mr rec-TM by alkaline β-elimination as well as chondroitin sulphate A also accelerated the inactivation of scu-PA by thrombin,

in agreement with a recent paper of Molinari *et al.* (1992). However, on a carbohydrate weight basis, 1000-fold more free CSGAG was required than intact high-Mr rec-TM to obtain the same effect. Reconstitution experiments showed that the addition of CSGAG to low-Mr rec-TM did not achieve the accelerating activity of high-Mr rec-TM. This indicates that the CSGAG should be linked to the TM polypeptide chain for the expression of the full activity of TM.

The accelerating effects of both high-Mr and low-Mr rec-TM were dose-dependent and showed an apparent Kd for their complexes with human thrombin of 0.4 and 14 nM respectively, somewhat lower than the Kds for bovine thrombin of 3 and 16 nM respectively (Parkinson *et al.*, 1990). It can be concluded that the CSGAG on TM plays a pivotal role in the acceleration of the inactivation of scu-PA by thrombin, both by providing a secondary binding site for thrombin (Bourin *et al.*, 1988; Preissner *et al.*, 1990), leading to a higher affinity for thrombin, and by increasing the activity of the TM-thrombin complex towards scu-PA.

The action of TM in the scu-PA inactivation reaction is probably based on induction of a conformational change in thrombin upon binding to the epidermal growth factor modules 5 and 6 of TM (Musci *et al.*, 1988). Additional conformational change in thrombin caused by binding to the secondary binding site on the GSGAG moiety (Bourin *et al.*, 1988; Preissner *et al.*, 1990) might occur. An additional explanation for the pivotal role of the GSGAG could be that the CSGAG provides a binding site for high molecular weight and low molecular weight scu-PA, resulting in a cyclic ternary complex between TM, thrombin and scu-PA. The idea of direct binding between TM and scu-PA is supported by the finding that active site-blocked or thrombin-derived tcu-PA can inhibit the TM acceleration of the inactivation of scu-PA (Molinari *et al.*, 1992). On the other hand, in the inactivation of thrombin by antithrombin III, which is accelerated by TM, the CSGAG moiety is also essential (Preissner *et al.*, 1990; Bourin & Lindahl, 1990; Koyama *et al.*, 1991*a*), but binding of antithrombin III to TM could not be detected (Preissner *et al.*, 1990).

If TM binds scu-PA directly, the model used for the estimation of the dissociation constants of the TM-thrombin complex would be inappropriate. However, as the Kds obtained with rabbit TM (De Munk *et al.*, 1991) and with high-Mr rec-TM (Fig. 2A) were very close to 0.5 nM, a value obtained in the literature by independent methods (Owen & Esmon, 1981; Haley *et al.*, 1989), the putative binding between TM and scu-PA is probably weak with regard to the strong binding between TM and thrombin and does not significantly affect the estimation of the Kd of the latter interaction.

In addition to the elucidation of some structure-function relationships in TM, this study may have interesting physiological implications. As discussed previously (Koyama

et al., 1991*a*; Parkinson *et al.*, 1992), glycosylation of TM in vivo may vary in a species-, tissue- or organ-specific manner. By modulating the CSGAG on TM, cells could regulate their scu-PA-inactivating potential and thereby u-PA activity-dependent processes, such as fibrinolysis as well as cell migration, tissue destruction and remodelling (De Munk *et al.*, 1991). Finally, potential antifibrinolytic effects of TM molecules should be considered when they are tested for anti-thrombotic therapy (Nawa *et al.*, 1992).

6.5 Acknowledgements

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CHAPTER 7

COMPARISON OF THE IN VITRO FIBRINOLYTIC ACTIVITIES OF LOW AND HIGH MOLECULAR WEIGHT SINGLE-CHAIN UROKINASE-TYPE PLASMINOGEN ACTIVATOR

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Summary

The fibrinolytic activity of low molecular weight (LMW) single-chain urokinase-type plasminogen activator (scu-PA) lacking the epidermal growth factor domain and the kringle domain was compared with the activity of high molecular weight (HMW) scu-PA. LMW scu-PA was 1-5 times less active than HMW scu-PA in a fibrin plate method, in a purified fibrin clot lysis assay and in a plasma clot lysis assay. Time course experiments in a chromogenic plasminogen activator assay suggested that LMW scu-PA was less sensitive to activation by plasmin than HMW scu-PA. This was confirmed in a scu-PA activation test, which showed that at a concentration of 40 IU/ml LMW scu-PA required a three-fold higher plasmin concentration for 50% activation in 20 min than did HMW scu-PA. Kinetic experiments in the presence of 0.1 M NaCl showed non-standard Michaelis-Menten kinetics for the activation by plasmin of both HMW and LMW scu-PA. In contrast, standard kinetics was observed at 0.15 M NaCl, showing a 2.6-fold lower catalytic efficiency for LMW scu-PA than for HMW scu-PA. It is concluded that the plasmin activation of LMW scu-PA is about 3 times slower than the activation of HMW scu-PA. This explains, at least partially, the lower fibrinolytic activity of LMW scu-PA in comparison with HMW scu-PA.

7.1 Introduction

Urokinase-type plasminogen-activator (u-PA) catalyses the conversion of the proenzyme plasminogen to the proteolytic enzyme plasmin (1-3). The single-chain form of u-PA (scu-PA) can be converted into an active two-chain form (tcu-PA) by a single cleavage between Lys 158 and Ile 159 by plasmin. A low molecular weight (LMW) form of scu-PA, lacking the epidermal growth factor domain and the kringle domain, has been obtained from monkey kidney cell cultures (4) and from the human lung carcinoma cell line CALU-3 (5). This enzyme is proteolytically derived from high molecular weight (HMW) scu-PA by the metalloprotease pump-1 (6,7) which cleaves between Glu 143 and Leu 144 (5). Recombinant LMW scu-PA variants have been made by deletion of Ser 1 to Glu 143 (8) or Cys 11 to Lys 135 (Δ125-scu-PA) (9).

LMW scu-PA variants might have several advantages over HMW scu-PA as a drug for thrombolytic therapy. The prime advantage is the smaller molecular weight of LMW scu-PA (32,000 as compared to about 50,000 for HMW scu-PA), which facilitates large-scale production. The second advantage is the absence of the growth factor domain which

is responsible for the interaction of HMW scu-PA with its specific high-affinity cell receptor (10). This interaction could possibly produce unwanted side-effects during thrombolytic therapy, because receptor-expressing cells are loaded with proteolytic activity. Moreover, it has recently been suggested that this interaction induces tyrosine phosphorylation and signal transduction (11), possibly affecting cell growth and differentiation (12-14).

Whether or not LMW scu-PA represents a potential thrombolytic agent, strongly depends on its fibrinolytic properties. It has been demonstrated that LMW scu-PA still possesses the fibrin-selective action of HMW scu-PA (5,15,16). The fibrinolytic potency of LMW scu-PA variants have been reported to be similar or lower than that of HMW scu-PA, depending on the test system and the preparations used (5,8,9,15-17).

An essential reaction in inducing fibrinolysis by scu-PA is the activation of scu-PA by plasmin. This reaction has recently been the subject of detailed kinetic analysis, which shows non-standard Michaelis-Menten kinetics with a relatively high activity of plasmin at low scu-PA concentrations (18,19). None of the above-mentioned studies on LMW scu-PA has investigated this aspect of the activation reaction. The aim of the present study was to compare the in vitro fibrinolytic properties of LMW scu-PA and HMW scu-PA thoroughly, including the activation reaction by plasmin.

7.2 Materials and methods

Proteins and Reagents

Human kidney cell culture HMW and LMW scu-PA (20,21) were a gift from Dr J. Henkin, Abbott Laboratories (Abbott Park, IL, U.S.A.), and were used throughout the study. In one experiment (Fig. 5, inset) additional preparations were used: HMW (22) and LMW (8) recombinant scu-PA provided by Dr H.R. Lijnen (Leuven, Belgium) and HMW scu-PA from a transformed kidney cell line, provided by Dr H. Bachmayer (Sandoz, Vienna, Austria). HMW and LMW tcu-PA were prepared by activation of the single-chain forms by plasmin (see below). Human plasmin, human glu-plasminogen, chromogenic substrates pyro-Glu-Gly-Arg-p-nitroanilide (S-2444) and D-Val-Leu-Lys-p-nitroanilide (S-2251) were from Chromogenix (Mölndal, Sweden). Plasminogen-rich bovine fibrinogen was prepared from bovine plasma (23). Radioiodine-labelled fibrinogen was from Amersham (Amersham, UK). Aprotinin was from Bayer AG (Leverkusen-Bayerwerk, Germany). BSA (A 7030) was purchased from Sigma Chemical CO. (St. Louis, MO, U.S.A.). Bovine thrombin was from Leo (Ballerup, Denmark) and ancrod from Knoll (Ludwigshafen, Germany). D-Val-Phe-Lys-chloromethyl ketone and D-Glu-Gly-Arg-chloromethyl ketone were from Calbiochem (La Jolla, CA, U.S.A.).

Concentrations of scu-PA and tcu-PA

Concentrations are expressed in International Units (IU) based on a comparison with the International Reference Preparation for tcu-PA (IRP code numbered 66/46). Preparations of scu-PA were first activated with plasmin (0.1 CU/ ml or 45 nM for 20 min at 37°C). The activities were compared in an amidolytic assay using 0.3 mM S-2444 in 0.05 M Tris/HCl, pH 8.8, 0.038 M NaCl, 0.01% Tween 80 and 20 KIU/ ml aprotinin. The specific activities of kidney cell culture HMW and LMW scu-PA amounted to 79,600 and 87,500 IU/mg, respectively.

Preparation of tcu-PA

HMW and LMW tcu-PA were prepared from the single chain forms by incubating them at a concentration of 100-10,000 IU/ml with 0.1 CU/ml (45 nM) plasmin at 37°C for 20 min. The plasmin was subsequently inhibited by incubation with 2 μM D-Val-Phe-Lys-chloromethyl ketone for 20 min at 37°C. Remaining inhibitor was removed by gel filtration.

Fibrin plate method

Fibrin plates were prepared by adding 10 μl 20 NIH U/ml thrombin or 30 U/ml ancrod to 6 ml plasminogen-rich bovine fibrinogen (24) in 0.05 M Veronal/HCl pH 7.75, 0.093 M NaCl, 1.6 mM $CaCl_2$ and 0.7 mM $MgCl_2$. After 3 hr at room temperature, 5 μl drops containing u-PA in 0.02 M Tris/HCl pH 7.5, 0.1 M NaCl and 0.01% Tween 80, were put on the fibrin plates. After 17 hr at 37°C the diameter of the lysis zones was measured.

Lysis of purified fibrin clots by incorporated u-PA

A turbimetric fibrinolysis assay (25) was performed with human fibrinogen after removal of residual plasminogen from the fibrinogen with a lysine-Sepharose column. In a well of a microtiter plate 140 μl fibrinogen (3.4 mg/ml) in 0.01 M Hepes, pH 7.4, 0.1 M NaCl and 0.01% Tween 80 was mixed with 20 μl u-PA (5-460 IU/ml), 20 μl Glu-plasminogen (1 μM) and 20 μl ancrod (50 U/ml) and incubated at room temperature. The optical density at 405 nm was measured every 3 min using an automatic Titertek multiskan (Eflab Oy, Finland).

Plasma clot lysis by scu-PA in the surrounding plasma

Radioiodine-labelled fibrin clot lysis was performed as follows: citrated human pooled plasma was mixed with ^{125}I-labelled human fibrinogen (final concentration 150,000 cpm/ml), then $CaCl_2$ (final concentration 20 mM) and thrombin (final concentration 6 NIH U/ml) were added. The mixture was aspirated into a 4-mm silicon tube and allowed

to clot at 37°C for 30 min. Pieces of 0.5 ml were cut, washed in 0.15 M NaCl and then incubated in 3 ml pooled plasma containing LMW or HMW scu-PA (final concentration 0-150 IU/ml) at 37°C for 6 hour. Every hour a 40 μl sample was taken and counted. The percentage of clot lysis was calculated from the amount of radioactivity released into the surrounding plasma.

Activation of glu-plasminogen

Glu-plasminogen (final concentration 0.3 μM, contaminating lysine was removed by gel filtration) was mixed with HMW or LMW scu-PA or tcu-PA (final concentration 6 IU/ml) and 0.35 mM S-2251 in 0.05 M Tris/HCl, pH 7.5, 0.1 M NaCl, 1.4 mg/ml polyethylene glycol and 0.01% Tween 80 in microtiter plates. During incubation at 37°C, the increase in optical density at 405 nm was measured in a Titertek multiskan..

Activation of scu-PA by plasmin

HMW and LMW scu-PA preparations (final concentration 40 IU/ml) were incubated with 0.01-100 mCU/ml (4.4 pM-44 nM) plasmin for 20 min at 37°C in 0.02 M Tris/HCl, pH 7.5, 0.1 M NaCl, 3mM $CaCl_2$, 0.1% Tween 80 and 0.1% BSA or in 0.05 M Tris/HCl, pH 8.8, 0.038 M NaCl and 0.01% Tween 80 (S-2444 assay buffer). The activation was stopped by diluting the mixture 5 times in the S-2444 assay buffer containing 20 KIU/ml aprotinin. Activated scu-PA was measured with S-2444 (final concentration 0.3 mM) as the increase in optical density at 405 nm at 37°C.

Kinetic experiments

Solutions of HMW and LMW scu-PA ranging from 7.4 to 270 nM were incubated with 0.11 mCU/ml (50 pM) plasmin in 0.02 M Tris/HCl, pH 7.5, 0.1 M NaCl, 3 mM $CaCl_2$, 0.1% Tween 80 and 0.1% BSA, at 37°C. Parallel incubations were performed in the same buffer containing 0.15 M NaCl instead of 0.1 M NaCl. Samples were taken every 7.5 min and 100 KIU/ml (final concentration) aprotinin was added to stop the reaction. Activated scu-PA was measured with S-2444 (final concentration 0.3 mM) in the presence of 100 KIU/ml aprotinin.

7.3 Results

To investigate the fibrinolytic properties of the HMW and LMW forms of scu-PA, three different global assays were used. First, the activities were measured on plasminogen-rich fibrin plates. Figure 1 shows that HMW and LMW scu-PA did not give parallel dose-

response curves. At low concentrations, LMW scu-PA was about 5 times less active than HMW scu-PA. At higher concentrations, however, they were equally active and reached the activities of the corresponding two-chain forms. HMW and LMW tcu-PA were equally active at all concentrations tested.

Secondly, the fibrinolytic activity of the four u-PA preparations incorporated in a fibrin clot was measured using a turbimetric method. In the presence of Glu-plasminogen and u-PA, purified fibrinogen was clotted with ancrod, to avoid inactivation of scu-PA by thrombin (26). The time required for 50% lysis was determined. We found parallel dose-response curves for all u-PA preparations (Fig. 2). LMW scu-PA was 5 times less active than HMW scu-PA. The two single chain-forms were significantly less active than the corresponding two-chain forms (4 and 14 times for HMW and LMW u-PA, respectively), whereas HMW and LMW tcu-PA had a more similar fibrinolytic activity.

In the third assay, plasma clots containing ^{125}I-fibrin were incubated in plasma with LMW and HMW scu-PA. The 50% lysis time of the two scu-PA preparations at five different concentrations was determined (Fig. 3). The fibrinolytic activity of LMW scu-PA was lower than that of HMW scu-PA, especially at low concentrations. The difference varied from 1.9 times at 50 IU/ml to 1.2 times at 150 IU/ml.

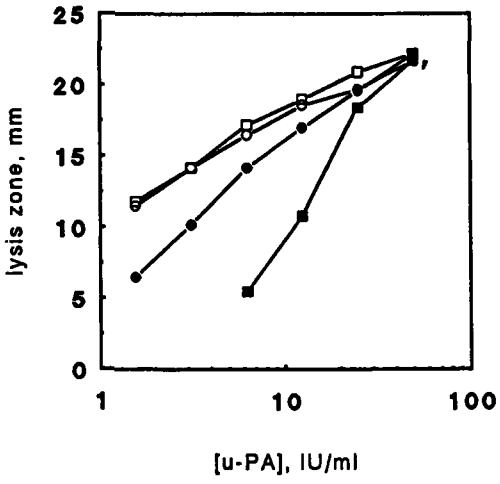

Figure 1. Activity on fibrin plates. Drops of 5 μl u-PA were incubated on plasminogen-rich bovine fibrin plates. After 17 hr at 37°C, the diameter of the lysis zone was measured. (•) HMW scu-PA, (○) HMW tcu-PA, (■) LMW scu-PA, (□) LMW tcu-PA.

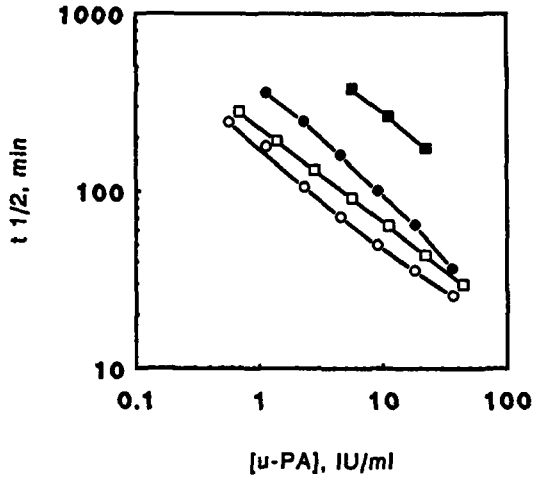

Figure 2. Lysis of purified fibrin clots. Human fibrinogen was clotted in microtiter plate wells in the presence of glu-plasminogen (0.1 μM final concentration) and u-PA. The optical density at 405 nm was measured every 3 min with a Titertek Multiskan, and the time required for 50% lysis was determined. (•) HMW scu-PA, (○) HMW tcu-PA, (■) LMW scu-PA, (□) LMW tcu-PA.

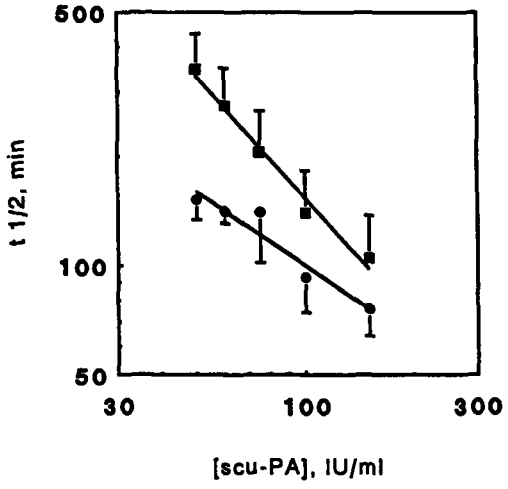

Figure 3. Plasma clot lysis. Plasma clots, labelled with ^{125}I-fibrin, were incubated in plasma containing HMW scu-PA (•) and LMW scu-PA (■). Labelled fibrin degradation products in the surrounding plasma were measured every hour and the time needed to lyse 50% of the clot was determined. Mean values ± s.e.m. were calculated from three independent experiments on three different days. HMW scu-PA appeared to be more active than LMW scu-PA at each concentration and on each day, indicating that the difference between HMW and LMW scu-PA was highly significant ($p < 0.01$, paired *t*-test).

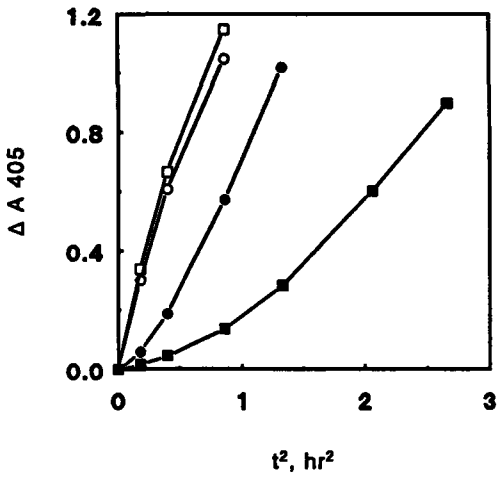

Figure 4. Activation of glu-plasminogen. The activation of glu-plasminogen (0.3 μM) by various forms of u-PA (0.6 IU/ml) was measured with simultaneously added chromogenic substrate for plasmin S-2251 (0.35 mM). (•) HMW scu-PA, (○) HMW tcu-PA, (■) LMW scu-PA, (□) LMW tcu-PA.

Lysis-versus-concentration curves of the purified fibrin clot lysis experiments and of the plasma clot lysis experiments (not shown) indicated that LMW scu-PA was less active than HMW scu-PA at all time points. Lysis versus-time-curves (not shown) demonstrated that clot lysis by LMW scu-PA had a longer lag phase than clot lysis by HMW scu-PA. To investigate the lag phases in more detail, LMW and HMW scu-PA and the corresponding two chain forms were incubated with Glu-plasminogen and S-2251 in a buffer without fibrin. Figure 4 shows plots of the change in absorbance at 405 nm against time square. As the slope of these plots at any time reflects the concentration of active plasminogen activator present at that time (27), it is clear that HMW tcu-PA was generated faster from the single chain precursor than LMW tcu-PA (closed symbols) This could be explained if HMW scu-PA were more sensitive for activation by plasmin than LMW scu-PA. To investigate this possibility, 40 IU/ml HMW and LMW scu-PA were incubated with various plasmin concentrations for 20 min at 37°C. Figure 5 shows that LMW scu-PA required a three-fold higher plasmin concentration for 50% activation than did HMW scu-PA. The inset of Fig. 5 shows that the difference was as much as 25-fold in a buffer with pH 8.8 and low ionic strength (S-2444 buffer). Under these conditions, the results obtained with LMW and HMW scu-PA from human kidney cell cultures were confirmed by data obtained with LMW and HMW recombinant scu-PA and with HMW scu-PA from a transformed human kidney cell line (Fig. 5, inset). To quantify the activation rates in more detail, we determined initial rates at varying scu-PA concentrations. The activation rates were constant during the first 30 min in which less than 2% scu-PA was activated. They were 1.8 to 3.2 times higher for HMW scu-PA than for LMW scu-PA. The initial activation rates and the scu-PA concentrations were used

to construct Lineweaver-Burk plots. Figure 6A shows, however, that non-linear plots were found, both with HMW and LMW scu-PA. As a recent paper reported that linear Lineweaver-Burk plots can be obtained at a physiological NaCl concentrations (28), The activation reactions were not only performed at 0.10 M NaCl, but also at 0.15 NaCl. Indeed, linear plots were obtained at 0.15 M NaCl, as shown in Fig. 6B. As the intercepts on the 1/S axis were small, the K_m values for HMW and LMW scu-PA far exceed the substrate concentration range tested (7-270 nM). The slopes of the curves correspond to catalytic efficiencies (k_{cat}/K_m) of 0.37 and 0.14 $\mu M^{-1}.s^{-1}$ for HMW and LMW scu-PA, respectively, pointing to a 2.6-fold difference in activatability.

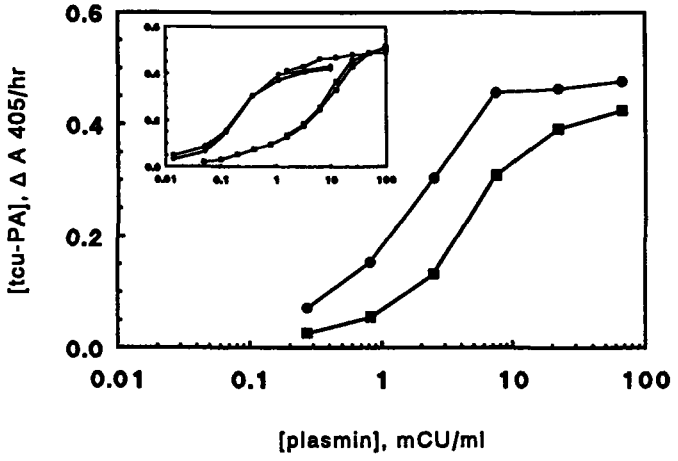

Figure 5. Activation of scu-PA by plasmin. HMW and LMW scu-PA preparations (40 IU/ml) were activated with different plasmin concentrations for 20 min at 37°C, in 0.02 M Tris/HCl, pH 7.5, 0.1 M NaCl, 3 mM $CaCl_2$, 0.01% Tween 80 and 0.1% BSA. The amount of generated tcu-PA was measured with S-2444. The inset shows scu-PA activation in 0.05 M Tris/HCl, pH 8.8, 0.038 M NaCl and 0.01% Tween 80. (•) HMW scu-PA and (■) LMW scu-PA from human kidney cell cultures; (○) HMW and (□) LMW recombinant scu-PA; (◊) HMW scu-PA from a transformed human kidney cell line.

7.4 Discussion

In order to evaluate the potential use of LMW scu-PA as a thrombolytic drug, we compared the fibrinolytic properties of LMW scu-PA and HMW scu-PA in three different global assays. Although each separate assay is, in principle, unsuited for comparing the activities of two different plasminogen activator species, the combined use of the three assays does afford information about the relative efficacy of LMW scu-PA and HMW scu-PA as fibrinolytic agents. The assays comprised a plasminogen-rich bovine fibrin plate

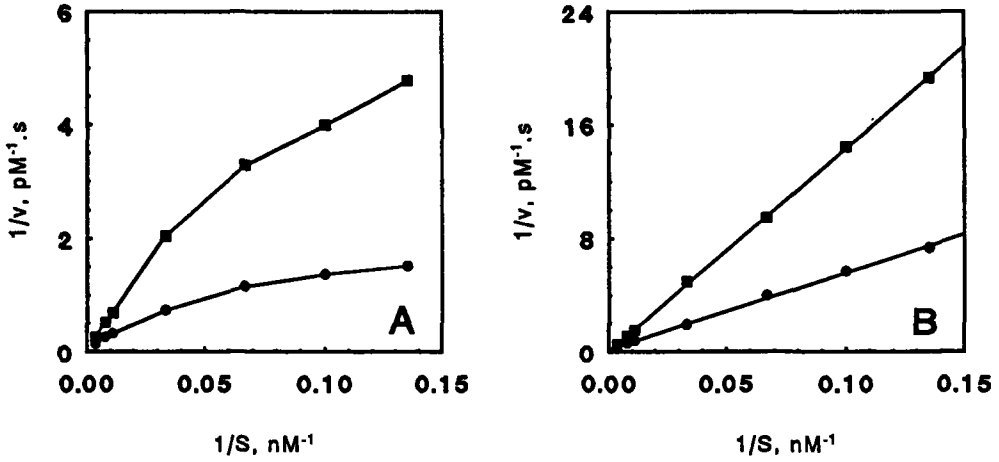

Figure 6. Lineweaver-Burk plots of the activation of scu-PA in the presence of 0.1 M NaCl (A) and 0.15 M NaCl (B). The rate of activation by plasmin (50 pM) was measured at varying concentrations of HMW (•) and LMW (■) scu-PA, as described in the Methods section. The straight lines in Fig. B were determined by using the least squares method.

method, a purified fibrin clot lysis assay (endogenous lysis) and a plasma clot lysis assay (exogenous lysis). In all three assays, LMW scu-PA appeared to be less active than HMW scu-PA. In two of these assays the difference between HMW and LMW scu-PA diminished at high concentrations of scu-PA.

It has been proposed that there is a sequence of three reactions involved in plasminogen activation by scu-PA (29):

$$\text{scu-PA} + \text{plasminogen} \rightarrow \text{scu-PA} + \text{plasmin} \quad \text{I}$$

$$\text{plasmin} + \text{scu-PA} \rightarrow \text{plasmin} + \text{tcu-PA} \quad \text{II}$$

$$\text{tcu-PA} + \text{plasminogen} \rightarrow \text{tcu-PA} + \text{plasmin} \quad \text{III}$$

In reaction I the low intrinsic activity of scu-PA activates a small amount of plasminogen. In reaction II, plasmin activates scu-PA to tcu-PA. The bulk of plasminogen is activated by tcu-PA with a relatively high catalytic efficiency in reaction III. Reaction III is probably not involved in the difference in fibrinolytic activity between HMW and LMW scu-PA, because no great difference was found in fibrinolytic activity of the two double-chain forms (Figs. 1-4). Although reaction I could play a role, the difference in activities can at least be partially ascribed to reaction II, as LMW scu-PA was more slowly activated by plasmin than was HMW scu-PA (Figs. 4-6). Considerably higher plasmin concentrations were required for LMW scu-PA than for HMW scu-PA in order to achieve

50% activation in 20 min (Fig. 5). Kinetic analysis showed that the catalytic efficiency of the activation of LMW scu-PA was 2.6 times lower than that of the activation of HMW scu-PA (Fig. 6).

A lower activation rate of LMW scu-PA with respect to HMW scu-PA was recently reported by Orsini et al. (9) for their Δ125-scu-PA variant (four-fold difference). The low sensitivity for plasmin could be associated with the resistance of this specific form to inactivation by thrombin and the thrombin-thrombomodulin complex (30,31). The present study shows, however, that the resistance to activation by plasmin is a general property of LMW scu-PA variants.

Another remarkable phenomenon, only detectable when a broad substrate concentration range is investigated in the presence of sub-physiological NaCl concentrations, is the non-standard Michaelis-Menten kinetics of the activation reaction of HMW scu-PA with plasmin which was called negative cooperativity (18,19). In the present study the activation of LMW scu-PA also seems to show this effect. The negative cooperativity has been ascribed to a secondary interaction between scu-PA and a lysine binding site on kringle 1-4 of plasmin, which is unfavourable for the activation reaction (18,19). It can be concluded from the results with LMW scu-PA that the NH_2-terminal part of scu-PA does not play a role in this interaction.

Corresponding to Lenich et al. (28), standard Michaelis-Menten kinetics was observed at 0.15 M NaCl (Fig. 6). Comparison of the data obtained at 0.10 and 0.15 M NaCl (Figs. 6A and 6B) indicates that the kinetics at 0.15 M NaCl resembles the slow kinetics at high substrate concentrations observed at 0.10 M NaCl. This suggests that, at 0.15 M NaCl, the secondary interaction between HMW or LMW scu-PA and plasmin occurs at all substrate concentrations tested.

Different theories have been put forward to explain the fibrin selective action of scu-PA (reviewed in 3). One theory proposes that, in reaction I, scu-PA has a higher activity towards fibrin-bound Glu-plasminogen than towards circulating Glu-plasminogen (32). Another theory, which may be supplementary to the previous one, is based on the well-established finding that plasmin bound to a fibrin clot is less accessible to α_2-antiplasmin than plasmin in the circulation, leading to a preferential activation of scu-PA (reaction II) on the clot surface (33,34). A third theory, which suggests accumulation of scu-PA on the clot surface by a direct binding phenomenon, has not been widely accepted. Recently this theory has, however, received a new impulse from the aforementioned studies on negative cooperativity (19). Longstaff et al. have found that scu-PA not only binds to the lysine-sensitive effector site on plasmin, but also to the corresponding site on plasminogen. Calculations suggest that in plasma, scu-PA will be 96% complexed with plasminogen. When plasminogen is bound by fibrin through lysine binding sites, the scu-PA-

plasminogen complex would dissociate and scu-PA is delivered on the fibrin surface (19). Our findings that the activation of LMW scu-PA also showed the non-standard Michaelis-Menten kinetics, suggest that this theory should also hold for LMW scu-PA. Indeed, LMW scu-PA has shown a fibrin selective action, both in vitro (5,15) and in vivo (16).

In conclusion, the lower level of activity of LMW scu-PA, in comparison with HMW scu-PA in various fibrinolysis assays can, at least partially, be ascribed to the lower activation rate of LMW scu-PA (reaction II). The reduction of the difference in fibrinolytic activity at higher scu-PA concentrations suggests that reaction II becomes less rate-limiting under those conditions. Clinical studies are required to evaluate the relative potency of LMW scu-PA under thrombolytic therapy conditions.

7.5 Acknowledgements

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CHAPTER 8

IN VIVO AND IN VITRO INTERACTION OF HIGH AND LOW MOLECULAR WEIGHT SINGLE-CHAIN UROKINASE-TYPE PLASMINOGEN ACTIVATOR WITH RAT LIVER CELLS

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Summary

The plasma clearance and the interaction of high (HMW) and low (LMW) molecular weight single-chain urokinase-type plasminogen activator (scu-PA) with rat liver cells was determined.

^{125}I-labeled HMW and LMW scu-PA were rapidly cleared from plasma with a half-life of 0.45 min and a maximal liver uptake of 55% of the injected dose. Liver uptake of scu-PA was mediated by parenchymal cells. Excess of unlabeled HMW scu-PA reduced the liver uptake of ^{125}I-HMW scu-PA strongly. In vivo liver degradation of scu-PA was reduced by inhibitors of the lysosomal pathway.

A high affinity binding site (Kd 45 nM, Bmax 1.7 pmol/mg cell protein) for both HMW and LMW scu-PA was determined on isolated parenchymal liver cells. Cross competition binding studies showed that LMW and HMW scu-PA bind to the same site. Tissue-type plasminogen activator, mannose- or galactose-terminated glycoproteins did not affect the scu-PA binding to parenchymal liver cells.

It is concluded that LMW and HMW scu-PA are taken up in the liver by a common, newly identified recognition site on parenchymal liver cells and are subsequently degraded in the lysosomes. It is suggested that this site is important for the regulation of the turnover of scu-PA.

8.1 Introduction

Plasminogen activators are proteolytic enzymes that convert plasminogen to plasmin. Plasmin is a broad spectrum protease that degrades fibrin and several proteins of the extracellular matrix. Because of their capacity to degrade fibrin via plasminogen activation, plasminogen activators are considered to be attractive thrombolytic agents. Two types of plasminogen activators (PA) are identified in mammals: tissue-type PA (t-PA) and urokinase-type PA (u-PA).

u-PA is produced as a single chain protein (scu-PA) with a molecular weight of 54,000 by e.g. kidney cells (1,2), many tumor cells (3) and fibroblasts (4). The pro-enzyme scu-PA is converted to two-chain u-PA (tcu-PA) by plasmin by a proteolytic cleavage between the Lys^{158} and Ile^{159}. A proteolytic derivative of scu-PA (LMW scu-PA), that lacks the first 143 N-terminal amino acids, has been isolated (2,5) and might be used for thrombolytic therapy. The HMW u-PA molecule contains three domains: a growth factor domain, a kringle domain and a protease domain. These three domains are highly homologous to the comparable domains in the t-PA molecule (6). u-PA has however a significantly longer

connecting peptide between kringle and protease domain than t-PA.

The use of scu-PA as a thrombolytic agent is, like the use of t-PA, hampered by a short plasma half-life (7-12). The liver and to a lesser extend the kidneys have been identified to play a major role in the organ uptake of scu-PA (both HMW and LMW scu-PA) in rabbits and monkeys (7-10). The receptor systems in liver and kidneys, responsible for the clearance of scu-PA from the blood are unidentified, but activation to two-chain u-PA appeared unnecessary (11). A receptor for u-PA has been described on monocytes and tumor cells (see for review Blasi (13)). This u-PA receptor is a heavily glycosylated protein with a Mr of 55,000-60,000 (14-16), which is anchored in the cell membrane by a glycolipid (17). The receptor binds single and two chain HMW u-PA with a high affinity (K_d 0.5-3 nM), but lacks affinity for LMW u-PA (18,19). Binding of HMW scu-PA to this receptor is not coupled to internalization and degradation (18,19). Recently it was shown that interaction of PAI-1 or PAI-2 with receptor bound two-chain u-PA leads to internalization and proteolytic degradation of both u-PA and PAI (20,21).

In the present study plasma clearance, organ uptake and mechanism of liver uptake of both high and low molecular weight scu-PA was studied in rats. In rat liver, the main site for scu-PA uptake, a binding site common for HMW scu-PA and LMW scu-PA is described on parenchymal liver cells. Binding of both types of scu-PA leads to proteolytic degradation via the lysosomal route.

8.2 Materials and methods

Materials

High molecular weight (HMW) and low molecular weight (LMW) single chain urokinase-type plasminogen activator (scu-PA) from human embryonic kidney cell cultures were gifts from Abbott (Abbott Park, Ill). Collagenase (type I), fetuin, neuraminidase (agarose bound), and bovine serum albumin (BSA) were from Sigma (St.Louis, Mo.). Collagenase (type D) and pronase were from Boehringer Mannheim (Mannheim, FRG). Recombinant t-PA was from Boehringer Ingelheim. Ovalbumin was from Serva Feinbiochemica (Heidelberg, FRG). Culture medium Ham's F-10 was from Gibco (Hoofddorp, The Netherlands). ^{125}I (carrier free) was from Amersham (Buckinghamshire, UK). Nycodenz was from Nycomed A/S (Oslo, Norway). 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid (HEPES) was from Merck (Darmstadt, FRG). Sephacryl S-200 HR was from Pharmacia LKB Technology AB (Uppsala, Sweden).

Lactosylated bovine serum albumin was made by reduction of the Schiff's base with cyanoborohydride. Asialofetuin was enzymatically prepared as described (22).

Radiolabeling of scu-PA

HMW scu-PA and LMW scu-PA were iodinated as described earlier for tissue-type plasminogen activator (22) by the iodogen method, resulting in a specific radioactivity between 3500 cpm/ng (LMW scu-PA) and 5000 cpm/ng (HMW scu-PA).

Purity of labeled scu-PA preparations was checked by SDS/PAA-gelelectrophoresis, under reducing and non-reducing conditions. Under both conditions HMW scu-PA and LMW scu-PA were identified as single bands with a Mr of 55,000 and 33,000, respectively. This indicates that after labeling the urokinase-type plasminogen activators have preserved their single-chain form.

Gel-filtration of the labeled proteins was performed on a calibrated Sephacryl S-200 HR column using a buffer (0.3 M NaCl, 10 mM Tris/HCl, pH 7.4) containing 0.1% (w/v) BSA, as a eluent. The labeled proteins eluted from the column as a single peak. From the retention time of the respective peaks it could be determined that the labeled proteins eluted under these conditions in their monomeric form.

The latent amidolytic activities of the labeled scu-PA preparations, determined on pyro-Glu-Gly-Arg-p-nitroanilide (S-2444) after activation of scu-PA with plasmin (23), were 90% of the unlabeled proteins. This shows that the preparations could still be activated and had intact active sites.

In vivo plasma clearance and organ uptake

12 weeks old male Wistar rats (225-275 g), used throughout the study, were anaesthetized by intraperitoneal injection with 20 mg of pentobarbital. The abdomen was opened, radiolabeled compounds (400 ng/kg rat) were injected via the vena penis and at the indicated times 0.3 ml blood samples were taken with heparinized syringes from the vena cava and liver lobules were tied off. Body temperature of the rats was kept at 36.5-37°C, using an infrared lamp. Blood samples were centrifuged for 2 min at 10,000 x g and 10% trichloroacetic acid-precipitable and 10% trichloroacetic acid-soluble radioactivity was determined in aliquots of plasma. Excised liver lobules (totally less than 10% of total liver weight) were weighed and radioactivity was counted. Total liver uptake was determined by weighing the total liver at the end of the experiment. Uptake of ^{125}I-scu-PA in other organs was determined by weighing total organs and counting radioactivity. Uptake in the various organs was corrected for the amount of plasma in these organs.

Rats were pretreated with leupeptin (20 mg/kg body weight) by intraperitoneal injection 60 minutes before ligand injection and with chloroquine (12.5 mg/kg body weight) by intraperitoneal injection 120 and 60 minutes before ligand injection.

Cell isolation procedures

For the determination of the contribution of different liver cell types to total liver uptake rats were anaesthetized and injected with ^{125}I-HMW scu-PA and ^{125}I-LMW scu-PA (400 ng/kg rat) via the vena penis. After 10 min the vena porta was cannulated and a liver perfusion at low temperature (< 8°C) was started using Hanks' buffer (supplemented with 10 mM Hepes). Parenchymal liver cells were separated from non-parenchymal liver cells after collagenase (0.05% (w/v) type I) perfusion of the liver at 8°C. To separate endothelial liver cells from Kupffer cells, the liver residue obtained after collagenase perfusion, was further digested by stirring with pronase (0.25%, w/v) for 20 min at 4°C, and separated using counterflow centrifugation, exactly as described earlier (22,24,25). The contribution of the various liver cell types to total liver uptake was calculated as described (22,24-26). As found for a number of substrates (22,24-26) no loss of cell-bound label and/or formation of acid-soluble radioactivity occurred during the low temperature cell isolation procedure, leading to a quantitative recovery of radioactivity associated with the isolated liver cells as compared to the total liver association. This was checked for each individual liver cell isolation by comparing the calculated liver association (from the relative contribution of the various cell types) and the determined total liver association.

In vitro binding studies

Parenchymal liver cells used for in vitro binding studies were isolated after perfusion of the rat liver for 10 min with collagenase (0.05% (w/v), type D) following the method of Seglen (27), modified as described previously (28). Purity of the cells was always more than 99%, while viability (checked by ATP content and trypan-blue exclusion) was more than 95% during the incubations. For binding studies with scu-PA, parenchymal cell suspensions were incubated at a density of 1 mg cell protein/ml Ham's F-10 medium, containing 2% (w/v) BSA and 25 mM HEPES (pH 7.4). Cell incubations were performed in Kartell plastic tubes at a circulating lab shaker (150 rpm, Adolf Kuhner) at either 4°C or 37°C. At the end of the incubations cells were washed at 4°C: two times with 10 mM tris/HCl, 150 mM NaCl, 5 mM $CaCl_2$ (pH 7.4) plus 0.2% BSA and once with the same buffer without BSA for 30 sec at 50 x g. Finally cells were resuspended in this buffer (without BSA) and cell-bound radioactivity was counted in a gamma counter, while cell protein was determined according to Lowry with BSA as standard. Dissociation of cell bound scu-PA was determined to be less than 3% during the time of washing procedure. The non-specific binding was determined in the binding studies as the residual binding of radiolabeled scu-PA to rat parenchymal liver cells in the presence of a 1000-fold unlabeled scu-PA.

Dissociation constant (Kd) and maximal binding (B_{max}) were determined from displacement curves according to a single site displacement model using a computerized non-linear fitting program (minimizing the sum of squares via the Simplex-iteration procedure) (Graph-Pad: H. Motulsky, ISI-Software) (29). Plasmaclearance curves were analyzed by computerized non-linear fitting following a bi-phasic clearance model using the same program.

8.3 Results

Plasma half-life and organ uptake of scu-PA

Both ^{125}I-LMW scu-PA and ^{125}I-HMW scu-PA showed after intravenously injection into rats a very similar pattern of plasma clearance and liver uptake (see Fig. 1). For both types of scu-PA a plasma half-life of 0.45 ± 0.04 min was found for the alpha phase, while the beta phase varied from 6.5 ± 0.9 min (HMW scu-PA) to 8.0 ± 1.2 min (LMW scu-PA). Maximal liver uptake was at 10 min after injection 56.5 ± 2.5% and 54.4 ± 3.3% for LMW scu-PA and HMW scu-PA, respectively. From 15 to 30 min after injection a decrease in liver associated radioactivity of scu-PA was observed, while in the same time an increase was found in trichloroacetic acid-soluble radioactivity in plasma. This sequence of events is consistent with liver uptake of ^{125}I-HMW scu-PA and ^{125}I-LMW scu-PA followed by degradation and secretion of degradation products into the circulation.

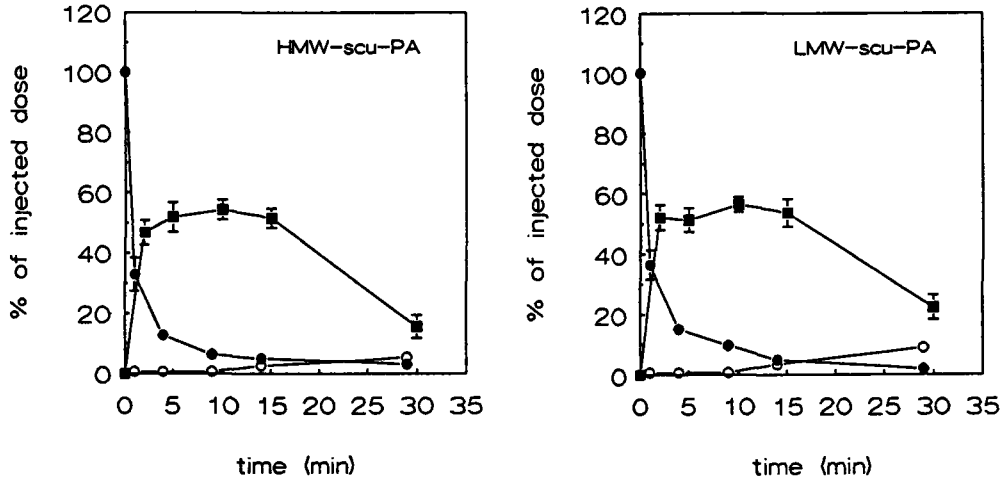

Figure 1. Plasmaclearance and liver uptake of ^{125}I-HMW scu-PA and ^{125}I-LMW scu-PA. At the indicated times after intravenous injection of ^{125}I-HMW scu-PA (left) or ^{125}I-LMW scu-PA (right) into male Wistar rats, plasma clearance and liver lobules (■) was determined. In plasma, both 10% trichloroacetic acid soluble (○) and precipitable (●) radioactivity was measured. Data are mean (n = 4) ± S.D.

The pathway of degradation of scu-PA in the liver in vivo was further investigated using the lysosomal inhibitors leupeptin and chloroquine (Fig. 2). Both inhibitors had no effect on the plasma clearance of either type of scu-PA (not shown). The initial uptake of scu-PA in the liver was also unaffected, but at 30 and 45 minutes after injection significantly higher levels of HMW scu-PA and LMW scu-PA were detected in the livers of animals, treated with protease inhibitors.

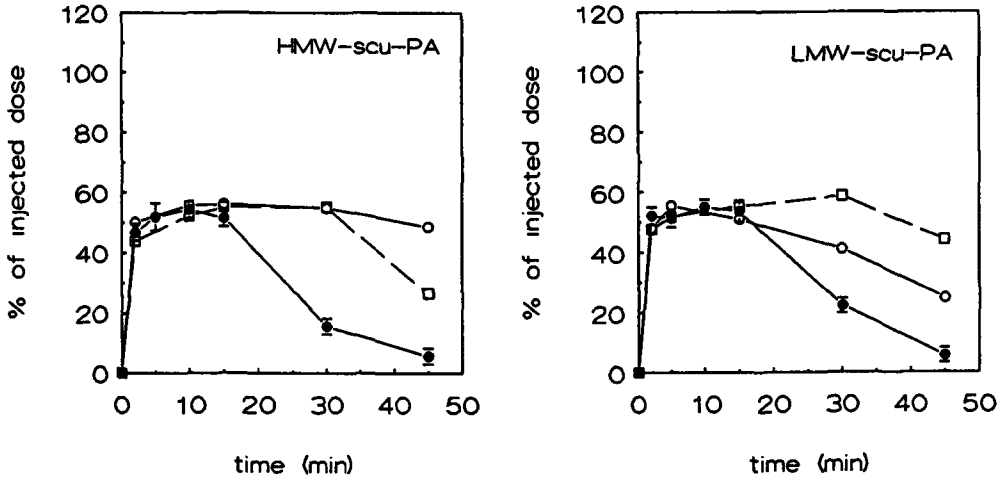

Figure 2. Effect of chloroquine and leupeptin on the liver association of ^{125}I-HMW scu-PA and ^{125}I-LMW scu-PA. Rats were pretreated with chloroquine (○), leupeptin (□) or not (•) as described in materials and methods. At the indicated times after injection of scu-PA liver lobules were tied off and radioactivity was counted.

The specificity of the interaction of ^{125}I-HMW scu-PA and ^{125}I-LMW scu-PA with the liver was determined by preinjection of rats with various ligands 1 min prior to injection of scu-PA. Ovalbumin (blocking the mannose-receptor) and asialo-fetuin (blocking the asialoglycoprotein receptor) (22) did not affect plasma clearance and liver uptake of HMW scu-PA and LMW scu-PA, indicating no involvement of these receptors in the uptake of both types of scu-PA by the liver (not shown). An intravenous injection of 20 mg unlabeled HMW scu-PA/kg body weight 1 min prior to injection of ^{125}I-HMW scu-PA, however, did inhibit the plasma clearance of ^{125}I-HMW scu-PA (Fig. 3). Concurrently initial liver uptake of ^{125}I-HMW scu-PA was reduced at 2 and 5 min after injection to 31.0% and 56.3% of the uptake in control rats, respectively.

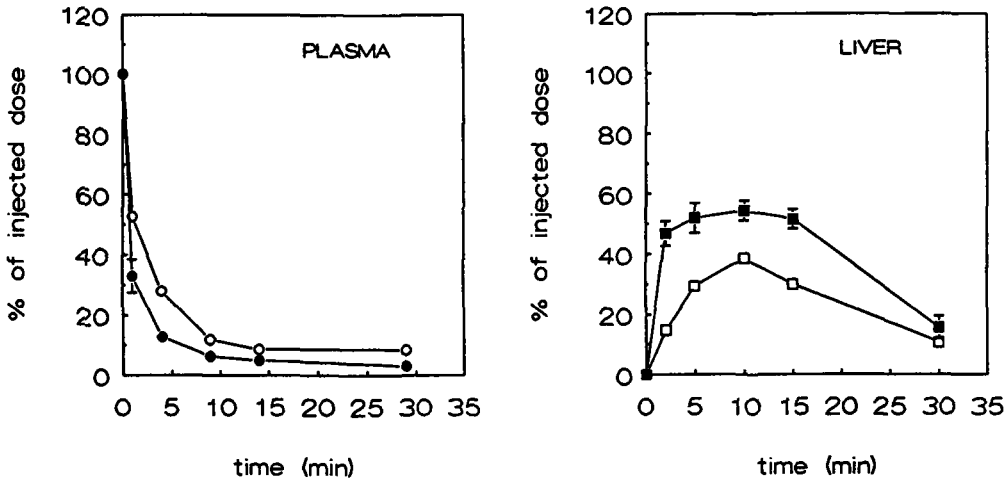

Figure 3. Effect of preinjection of unlabeled HMW scu-PA on the plasmaclearance and liver uptake of ^{125}I-HMW scu-PA. Rats were injected intravenously with 20 mg HMW scu-PA (open symbols) per kg body weight 1 min prior to injection of ^{125}I-HMW scu-PA or not pre-treated (closed symbols). At the indicated times after injection of the radiolabeled ligand liver uptake (■,□) and plasmaclearance (●,○) was determined. Bars represent S.D. of three experiments.

It appeared that in addition to the liver other sites in the body must be involved in the plasmaclearance of scu-PA. Therefore we determined the relative contribution of other organs to the plasma clearance of scu-PA (Fig. 4). Besides the liver (51.3 ± 4.3%), kidney (12.9 ± 1.3%), skin (6.0 ± 0.6%) and muscles (10.3 ± 1.0%) contributed to the plasma clearance of ^{125}I-LMW scu-PA. For ^{125}I-HMW scu-PA these values were: liver (55.2 ± 2.5%), kidneys (9.5 ± 2.6%), skin (2.3 ± 1.2%) and muscles (3.2 ± 0.4%). The uptake of ^{125}I-LMW scu-PA by skin and muscles was significantly larger than that of ^{125}I-HMW scu-PA.

The capacity of other organs than the liver to interact with scu-PA was studied in rats in which, by complete hepatectomy, the liver did not contribute to the plasma clearance. For HMW scu-PA an increased plasma half-life was found and 30 min after injection of the ligand still 37% of the injected dose was present in the circulation (Fig. 5). The tissue uptake of HMW scu-PA was performed, in the absence of liver uptake, by kidneys (32.9 ± 2.9%), muscles (5.6 ± 0.2%) and skin (16.9 ± 0.3%). More than 90% of the injected dose was present in the aforementioned tissues plus plasma and no trichloroacetic acid-soluble radioactivity was found in the plasma at 30 minutes after injection. This indicates that little or no degradation was mediated by extra-hepatic organs. For ^{125}I-LMW scu-PA also a prolonged plasma half-life was found after

hepatectomy and 30 minutes after injection 27.2 ± 1.9% of the injected dose was recovered in the plasma. At this time 38.8 ± 4.2% of the injected dose of LMW scu-PA was recovered in the skin, while kidneys (18.0 ± 0.7%) and muscles (7.9 ± 2.2%) also contributed substantially to the plasma clearance of LMW scu-PA. Again more than 90% of the radiolabel was present in the tissues shown and no trichloroacetic acid-soluble radioactivity was found in the plasma, indicating that degradation during hepatectomy was absent.

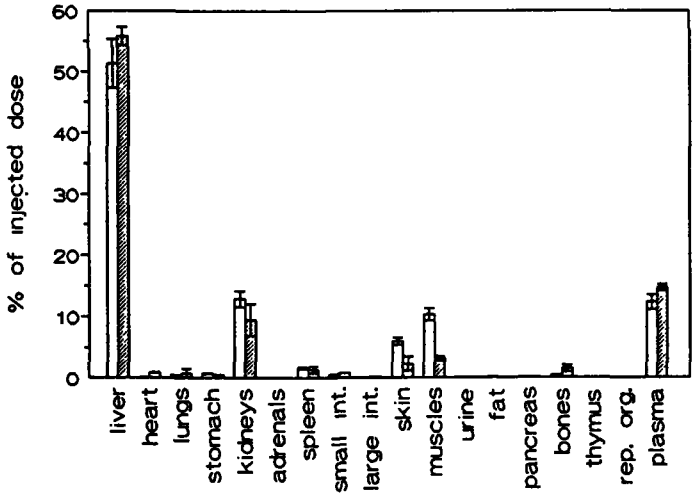

Figure 4. Tissue distribution of intravenously injected ^{125}I-HMW scu-PA or ^{125}I-LMW scu-PA. At 5 minutes after intravenous injection of ^{125}I-HMW scu-PA (hatched bars) or ^{125}I-LMW scu-PA (open bars) into rats the amount of radioactivity in the various tissues was determined. Recovery of the injected amount of radioactivity in the tissues shown was 92.1 ± 5.3% for HMW scu-PA and 97.5 ± 3.5% for LMW scu-PA (n = 3, mean ± S.D.).

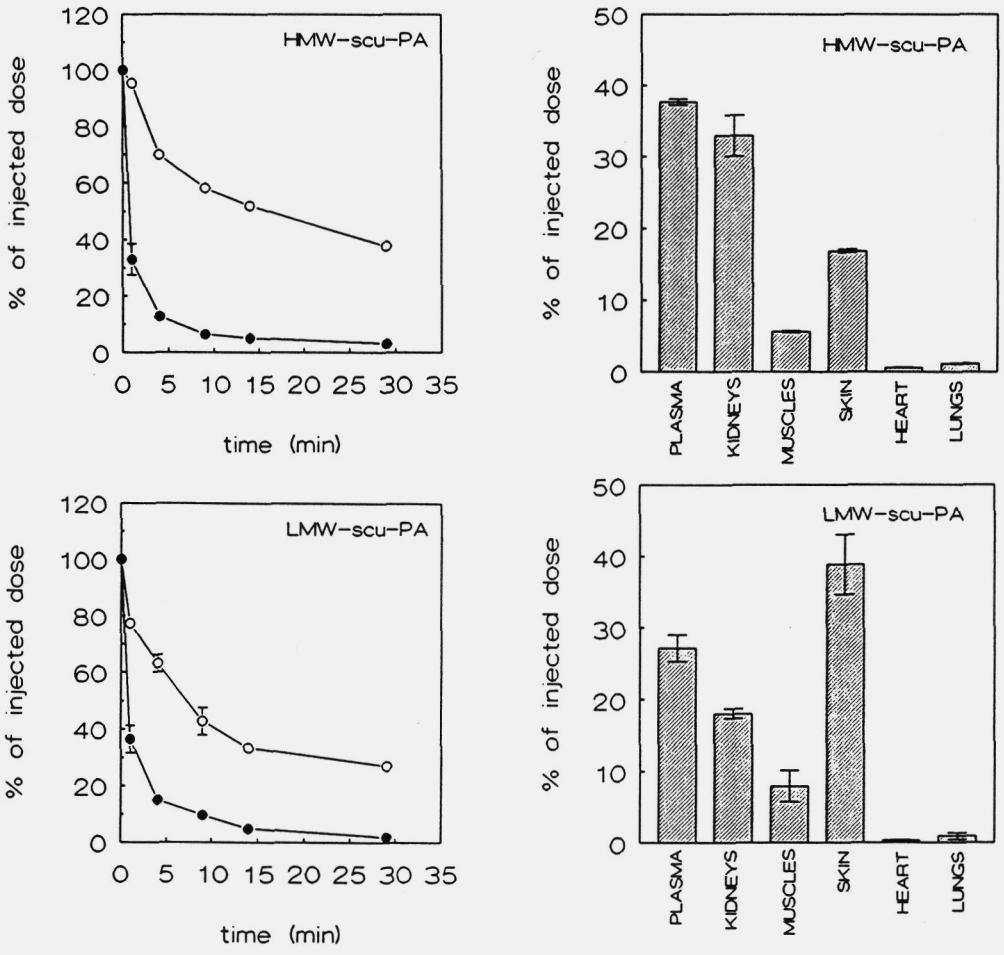

Figure 5. Tissue distribution and plasmaclearance of intravenously injected ^{125}I-HMW scu-PA and ^{125}I-LMW scu-PA in hepatectomized rats. Rats were hepatectomized and subsequently injected with radiolabeled HMW scu-PA (top) or LMW scu-PA (bottom). At the indicated times blood samples were taken and radioactivity (open symbols) was counted in aliquots of plasma. Control plasmaclearance is shown by the closed symbols. At 30 min after injection rats were sacrificed and radioactivity was counted in the indicated tissues.

Liver cell distribution of scu-PA

Table 1 shows the contribution of the various liver cells types to the total liver uptake of ^{125}I-scu-PA. 10 min after injection of ^{125}I-scu-PA the various liver cell types were isolated and it was found that parenchymal liver cells were responsible for about 90% to

the total liver uptake of both HMW scu-PA and LMW scu-PA. Non-parenchymal liver cell types contributed only about 10% to the total liver uptake of scu-PA.

Table 1. Relative contribution of the different liver cell types to liver uptake of scu-PA.
10 minutes after intravenous injection of ^{125}I-LMW and ^{125}I-HMW scu-PA a liver cell isolation at 8°C was started. Multiplication of the % of the injected dose/mg cell protein (not shown) with the amount of protein that each liver cell type contributes to total liver protein, results in the scu-PA uptake (expressed as % of total liver) by each cell type. Recovery of the injected dose in the different liver cell types was 112.1 ± 11.6% (HMW) and 105.1 ± 4.0% (LMW). Data represent the mean of three experiments ± S.D.

Cell type	^{125}I-LMW scu-PA	^{125}I-HMW scu-PA
parenchymal	87.7 ± 4.6	89.2 ± 4.1
endothelial	7.2 ± 2.3	6.1 ± 1.6
Kupffer	5.1 ± 2.4	4.9 ± 2.8

In vitro interaction of scu-PA with liver cells: binding and competition studies

Since parenchymal liver cells were responsible for 90% of the liver uptake of HMW scu-PA and LMW scu-PA, the specificity of the interaction of the two types of scu-PA with this liver cell type was studied in vitro.

Figure 6 shows the displacement of ^{125}I-HMW scu-PA with increasing amounts of unlabeled HMW scu-PA after a 2 hours incubation at 4°C. After 2 hours maximal binding had been reached and the level of binding was identical to that reached after 20 min incubation at 37°C (not shown). From the displacement curves the apparent K_d for the binding of HMW scu-PA to parenchymal liver cells was 45 nM, while maximally 1.80 ± 0.11 pmol/mg cell protein (or about 800,000 binding sites per cell) was bound. For ^{125}I-LMW scu-PA a similar inhibition experiment was performed (Fig. 6), which indicated that LMW scu-PA bound specific to parenchymal liver cells with a K_d of 45 nM and maximal binding of 1.56 ± 0.11 pmol/mg cell protein.

Since ^{125}I-HMW scu-PA was used at a concentration at which the u-PA-receptor on monocytes and tumor cells is reported to be saturated (13), a binding curve was determined at a concentration range from 0-1 nM HMW scu-PA (Fig. 7). It is clear that in this concentration range no saturation of the binding was observed.

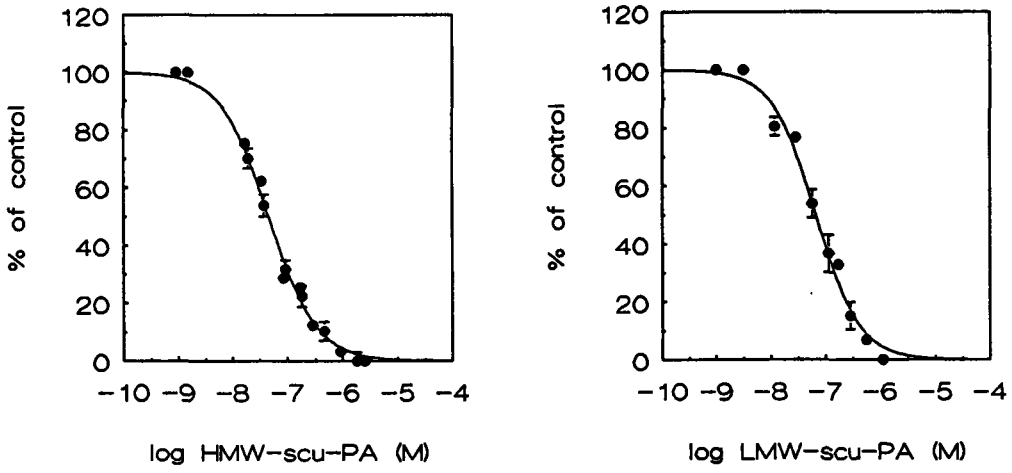

Figure 6. Effect of unlabeled scu-PA on the binding of ^{125}I-HMW scu-PA and ^{125}I-LMW scu-PA by rat parenchymal liver cells in vitro. Freshly isolated rat parenchymal liver cells were incubated for 2 hours at 4°C in the presence ^{125}I-HMW scu-PA (1.49 nM, left) or ^{125}I-LMW scu-PA (24.1 nM, right) with increasing of unlabeled HMW scu-PA and LMW scu-PA, respectively. At the end of the incubation the cell bound radioactivity was counted and amount of cell protein was determined. Data are expressed as % of specific binding. Total binding for HMW scu-PA was 2.9 ± 0.2 ng/mg cell protein and for LMW scu-PA 17.8 ± 1.1 ng/mg celll protein; aspecific binding was 9.3 ± 0.3% and 9.2 ± 0.3% of the total binding, respectively. Data are mean ± S.D. (n = 3).

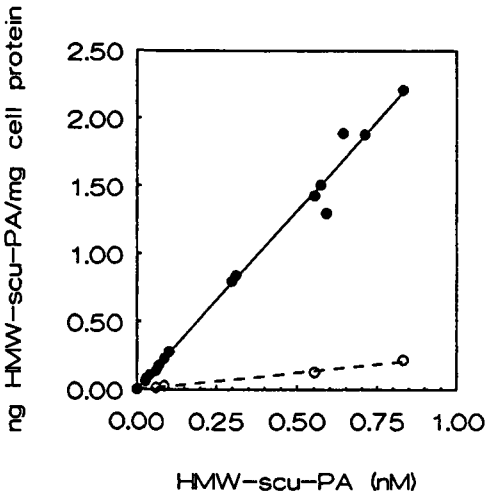

Figure 7. Binding of ^{125}I-HMW scu-PA to parenchymal liver cells as a function of the HMW scu-PA concentration. Increasing amounts of ^{125}I-HMW scu-PA were added to freshly isolated rat parenchymal liver cells in the presence (○) or absence (•) of 1 μM unlabeled HMW scu-PA. After two hours incubation at 4°C, cells were washed and the amount of radioactivity per mg cell protein was determined.

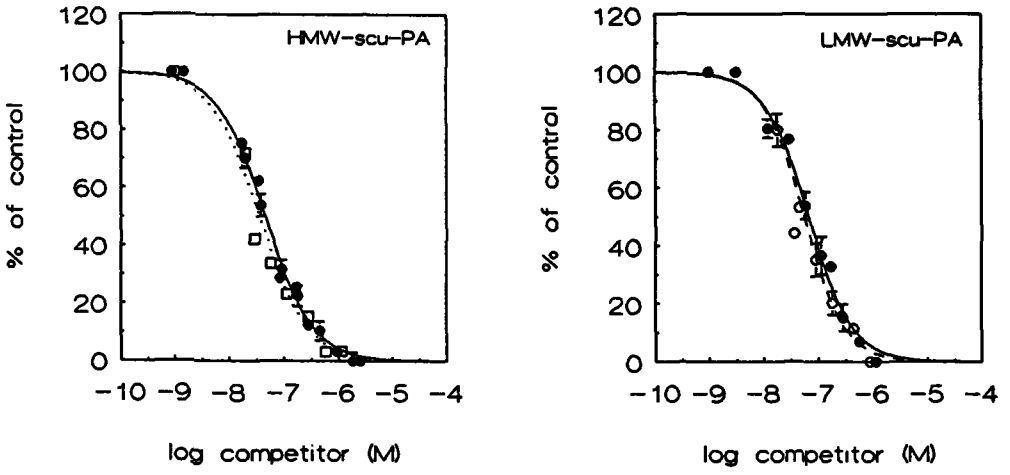

Figure 8. Effect of unlabeled scu-PA on the binding of ^{125}I-HMW scu-PA and ^{125}I-LMW scu-PA by rat parenchymal liver cells in vitro. Freshly isolated rat parenchymal liver cells were incubated for 2 hours at 4°C in the presence ^{125}I-HMW scu-PA (left) with increasing amounts of unlabeled HMW scu-PA (solid line) and LMW scu-PA (broken line) or parenchymal cells were incubated in the presence of ^{125}I-LMW scu-PA (right) with increasing amounts of unlabeled LMW scu-PA (solid line) and HMW scu-PA (broken line). At the end of the incubation cell bound radioactivity was counted and the amount of cell protein was determined. Data are expressed as % of specific binding. Values for total and non-specific binding are given in the legend of Fig. 6. Data are mean ± S.D. (n = 3).

The apparent absence of a monocytic type of receptor on rat parenchymal liver cells, which is specific for HMW scu-PA and does not bind LMW scu-PA, initiated further studies to the specificity of the binding site on parenchymal liver cells. The coincidence of K_d and maximal number of scu-PA molecules bound per parenchymal cells for HMW scu-PA and LMW scu-PA stimulated us to perform cross-competition experiments. Figure 8 shows that the binding of ^{125}I-HMW scu-PA to parenchymal liver cells can be equally well displaced by unlabeled HMW scu-PA and LMW scu-PA respectively. The binding of ^{125}I-LMW scu-PA to parenchymal liver cells can also be displaced to the same degree by equal molarities of LMW scu-PA or HMW scu-PA. The specificity of the binding of scu-PA to parenchymal liver cells was further determined by competition experiments with the related protein tissue-type plasminogen activator (t-PA), ovalbumin, asialo-orosomucoid and lactosylated albumin (Fig. 9). t-PA did not influence the binding of ^{125}I-HMW scu-PA or ^{125}I-LMW scu-PA, while unlabeled HMW scu-PA or LMW scu-PA had no effect on the binding of ^{125}I-t-PA by parenchymal liver cells (J. Kuiper, unpublished results). Lactosylated albumin or asialo-orosomucoid, which block in the applied range

the asialoglycoprotein receptor, did not affect the level of binding of ^{125}I-HMW scu-PA or ^{125}I-LMW scu-PA to parenchymal liver cells. The mannose-terminal glycoprotein ovalbumin had also no effect on the binding of ^{125}I-HWM-scu-PA and ^{125}I-LMW scu-PA.

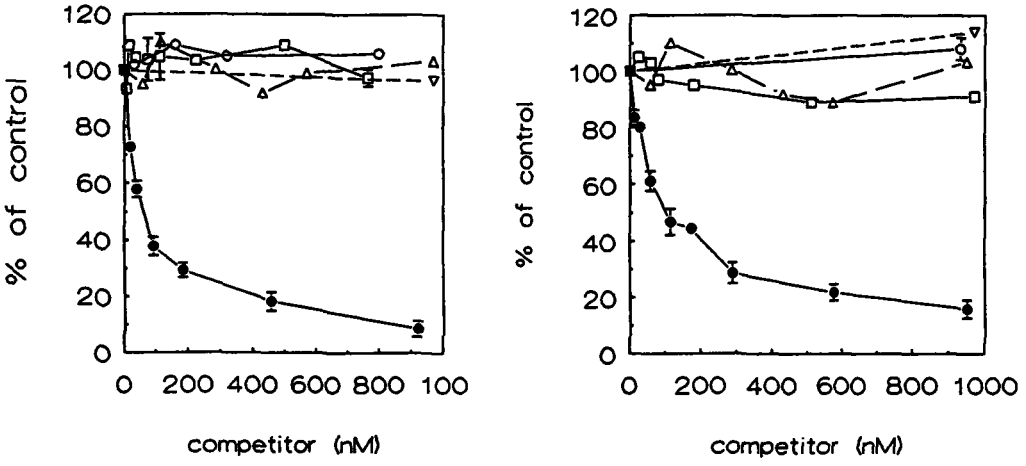

Figure 9. *Comparison of the ability of unlabeled proteins to compete with the binding of* ^{125}I-HMW scu-PA and ^{125}I-LMW scu-PA by rat parenchymal liver cells in vitro. Freshly isolated rat parenchymal liver cells were incubated for 2 hours at 4°C in the presence ^{125}I-HMW scu-PA (left) and ^{125}I-LMW scu-PA (right) with increasing amounts of unlabeled HMW scu-PA (•, left) or unlabeled LMW scu-PA (•, right), recombinant tissue-type plasminogen activator (□), lactosylated bovine serum albumin (○), ovalbumin (▽) and asialo-orosomucoid (△). At the end of the incubation the radioactivity was counted and the cell protein was determined. Data are expressed as mean ± S.D. (n = 3).

8.4 Discussion

The presented data show that human LMW scu-PA and HMW scu-PA are cleared from the plasma in rats with very comparable kinetics, a plasma half-life for the alpha-phase of 0.45 min and for the beta-phase of 6-8 min. Both types of scu-PA are mainly taken up by the liver (approximately 55% of the injected dose). Besides the liver, the kidneys, skin and muscles contributed significantly to the plasma clearance of scu-PA. In rabbits and monkeys (8,9), like rats, the liver also played a major role in the plasmaclearance of human scu-PA. The resemblance between the pharmacokinetic behaviour of high- and low molecular weight scu-PA in rats is in agreement with the similar pharmacokinetic behaviour of LMW scu-PA and HMW scu-PA in rabbits and monkeys (8).

The cellular site of in vivo recognition of scu-PA within the liver was determined. Both for LMW scu-PA and HMW scu-PA 90% of the liver associated radioactivity was

recovered in the parenchymal liver cells. In vivo and in vitro association of ^{125}I-HMW scu-PA by the liver parenchymal cells could be inhibited by unlabeled HMW scu-PA. In vivo inhibition of liver uptake of ^{125}I-HMW scu-PA by unlabeled HMW scu-PA was less efficient than in vitro inhibition of ^{125}I-HMW scu-PA binding to parenchymal liver cells by unlabeled HMW scu-PA. This may be the consequence of the fact that in vivo, in contrast to in vitro, unlabeled scu-PA is metabolized and the concentration of competitor is rapidly decreasing. This may also explain in vivo the higher percentages of inhibition at the shortest times after injecion. A similar effect has been observed blocking the uptake of ^{125}I-t-PA in vivo by unlabeled t-PA (22).

The binding of HMW scu-PA to isolated parenchymal liver cells showed a K_d of 45 nM and a maximal binding of 1.8 pmol/mg cell protein was found, which is equivalent to about 800,000 binding sites/cell. For the binding of LMW scu-PA to parenchymal liver cells very similar binding characteristics were found. The apparent affinity of the parenchymal liver cell for HMW scu-PA is a factor 15 to 150 lower than the apparent affinity of several other cell types (monocytes, tumor cells) for HMW scu-PA (13), but higher than the affinity reported for rat parenchymal liver cells and highly labeled tcu-PA (K_d > 300 nM) (30). A unique feature of the parenchymal cell recognition site, which contrasts the monocytic u-PA receptor is the fact that the scu-PA receptor on liver cells recognizes both LMW scu-PA and HMW scu-PA with a similar affinity. The monocytic receptor recognizes the growth factor-domain in the HMW scu-PA molecule and therefore shows no affinity for LMW scu-PA (18,19). Binding of HMW u-PA to the monocytic u-PA receptor is reported to be strictly species specific: human u-PA did not bind to mouse monocytes, while murine u-PA did not bind to human monocytes (13). Species specificity for the organ uptake may be less strict, since, despite quantitative differences, in all species studied so far human scu-PA appears to be taken up predominantly by the liver and to a varying extend by the kidneys (7-12). Recently, a common binding site for HMW scu-PA and LMW scu-PA was also suggested by means of ligand blot studies to be present on human endothelial cells (31).

The kinetics of liver association of LMW scu-PA and HMW scu-PA are indicative for uptake coupled to degradation (8,11). Inhibitors of the lysosomal degradation route (chloroquine and leupeptin) inhibit the disappearance of radioactivity from the liver of both LMW scu-PA and HMW scu-PA, suggesting that degradation of scu-PA inside the liver is executed in the lysosomes. Rat parenchymal liver cells do not produce PAI-1 (32) nor possess m-RNA for PAI-1 or PAI-2 (33) and since in vitro HMW scu-PA and LMW scu-PA degradation by parenchymal liver cells was observed (J. Kuiper, unpublished results), it apparently occurs in the absence of PAI-1 or PAI-2. The degradation of both HMW u-PA and LMW u-PA in its single chain form contrasts degradation of HMW u-

PA by monocytes and tumor cells, which only internalize and degrade two-chain HMW u-PA after binding of PAI-1 or PAI-2 (20,21).

The specificity of the scu-PA interaction with parenchymal liver cells was indicated by the fact that the related protein t-PA did not affect the binding of HMW scu-PA or LMW scu-PA to parenchymal liver cells. This correlates with the finding that the hepatic uptake of t-PA and tcu-PA is mediated by distinct receptor systems (30). It may also suggest that the amino acids which differ between the rather homologous t-PA and u-PA molecules are involved in the parenchymal liver cell recognition. The binding and uptake of the glycoprotein scu-PA to parenchymal liver cells is not mediated by a galactose- or a mannose-specific receptor, since preinjection of glycoproteins (mannose- or galactose-terminated) does not affect in vivo hepatic uptake or in vitro parenchymal liver cell binding of LMW scu-PA or HMW scu-PA. This finding correlates well with conclusions, drawn from the use of non-glycosylated recombinant scu-PA in rabbits (8).

The prominent role of the liver in scu-PA clearance and degradation was further subscribed by performing experimental hepatectomy. In hepatectomized rats plasma clearance of LMW scu-PA and HMW scu-PA was significantly reduced. For HMW scu-PA hepatectomy resulted in a sharp increase in kidney uptake, whereas for LMW scu-PA hepatectomy resulted in an unexpected high uptake in the skin. In the absence of liver uptake the kidneys are able to bind high amounts of HMW scu-PA, but lack apparently the ability to bind LMW scu-PA. No degradation of both types of scu-PA is found in hepatectomized animals, which may indicate that the specific HMW scu-PA binding to the kidney does not lead to degradation of HMW scu-PA. The kidney binding site shares therefore recognition properties with the u-PA receptor on monocytes and tumor cells (13). Further studies on the metabolism of tcu-PA-PAI by kidneys are however needed for further identification of the kidney binding site. The high interaction of LMW scu-PA with the skin during hepatectomy may be due to extra-vascularization as a consequence of its low molecular weight, rather than a specific phenomenon.

It is concluded that rat parenchymal liver cells possess a common receptor for HMW scu-PA and LMW scu-PA. Binding leads to internalization and proteolytic degradation involving a lysosomal route. The relative importance of the newly identified u-PA receptor system in the liver remains to be elucidated, but it may be anticipated to play a role in the regulation of maintaining low plasma-levels of scu-PA (34).

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CHAPTER 9

GENERAL DISCUSSION

9.1 Introduction

This thesis focused on the fibrinolytic properties of low molecular weight (LMW) and high molecular weight (HMW) single-chain urokinase-type plasminogen activator (scu-PA or pro-urokinase), including some specific reactions which could influence the fibrinolytic activity of scu-PA.

LMW scu-PA was originally purified from monkey kidney cell conditioned medium (Wijngaards et al. 1983, 1986). Later on, LMW scu-PA was purified from the human lung carcinoma cell line CALU-3 (Stump et al. 1986). Subsequently, recombinant LMW scu-PA variants were produced in CHO cells (Lijnen et al. 1988) and Escherichia coli (Günzler et al. 1987, Orsini et al. 1991). We investigated urine as a potential source for the isolation of LMW scu-PA (chapter 3). The presence of LMW scu-PA in urine seemed likely because of the observation that cultured kidney cells can produce LMW scu-PA. Moreover, both single-chain and LMW forms of u-PA have been isolated from urine. We found however, that LMW u-PA was only present in some samples and then only in small amounts. In addition, only a quarter of total u-PA was in the single-chain form. It was concluded that urine is not a suitable source for for the isolation of LMW scu-PA. We therefore mostly used a LMW scu-PA preparation from human kidney cell cultures (a gift from Abbott Laboratories, Abbott Park Illinois, USA). This LMW scu-PA preparation has been proteolytically derived from HMW scu-PA by the metalloprotease Pump-1 (Marcotte et al. 1992).

LMW scu-PA could represent a promising thrombolytic drug. Like HMW scu-PA, it is a fast acting and effective agent, it is fibrin clot selective, it does not react with plasminogen activator inhibitors, and it is a human protein and probably non-antigenic. Moreover, it lacks the growth factor domain, and LMW scu-PA cannot be lost by binding to the u-PA specific receptor on cells (Asselbergs et al. 1993). Nor can it induce cellular

processes via binding to this receptor. Finally, it is possibly more efficiently produced by prokaryotic cells because it has only half of the twelve disulphide bridges. However, the exact molecular mechanisms of fibrinolysis by HMW and LMW scu-PA are poorly understood. The studies reported in this thesis describe: the occurrence of scu-PA and tcu-PA in urine and the inhibition of tcu-PA by Plasminogen Activator Inhibitor-3 (PAI-3), the role of heparin and other sulphated glycosaminoglycans in different interactions of scu-PA and tcu-PA, the inactivation of scu-PA by thrombin, the activation of scu-PA by plasmin, the in vitro fibrinolytic activity and the in vivo liver clearance of scu-PA. HMW and LMW scu-PA were compared throughout the studies.

9.2 Significance of the results for therapeutic and physiological thrombolysis

The glycosaminoglycan-dependent inhibition of tcu-PA by PAI-3 in urine is abolished at physiological salt concentrations (chapter 3). Higher concentrations of the reactants or other types of sulphated glycosaminoglycans than are present in urine may possibly overcome this salt effect. Complexes of tcu-PA and PAI-3 have been shown in plasma of patients injected with tcu-PA (Geiger et al. 1989). PAI-1 is the primary inhibitor of tcu-PA in plasma. When PAI-1 has been consumed by therapeutic PA concentrations, inhibition by PAI-3, which is present in plasma in a much higher concentration, can take place. The main function of PAI-1 in plasma and platelets is probably to protect a thrombus against premature lysis. It could be speculated that PAI-3 also protects a thrombus. In that case, activated protein C may stimulate fibrinolysis via neutralization of both PAI-1 and PAI-3.

In thrombolytic therapy, heparin is used to prevent reocclusion. Heparin enhances the inhibition of coagulation. It has been reported in a number of studies that heparin stimulates the activation of plasminogen by t-PA and tcu-PA. In chapter 4, it is pointed out that the activation of plasminogen by LMW and HMW tcu-PA is indeed stimulated by heparin, but only at sub-physiological salt concentrations. Heparin also stimulates inactivation of HMW and LMW scu-PA by thrombin (Molinari et al. 1992), which is always generated during thrombolytic therapy. Stimulation of the inactivation of HMW scu-PA is not abolished at physiological salt concentrations (unpublished results). Inactivation of LMW scu-PA is possibly stimulated to a lesser extent by heparin at physiological salt concentrations, due to the lower affinity of LMW scu-PA for heparin.

Heparin may affect the fibrinolytic system in yet another way. Both t-PA and HMW scu-PA bind to heparin and related sulphated glycosaminoglycans at physiological salt concentrations (chapter 4). One might therefore expect that t-PA and scu-PA in vivo are

bound to glycosaminoglycans on the endothelial surface. Infusion of heparin may liberate t-PA and scu-PA from the glycosaminoglycans and bring them into the circulation.

Thrombomodulin-accelerated inactivation of both HMW and LMW scu-PA by thrombin will probably occur during thrombolytic therapy (chapter 5 and 6). Although thrombin-cleaved scu-PA may have thrombolytic potency in vivo (Abercrombie et al. 1990, Collen et al. 1989), thrombolytic therapy with scu-PA will possibly be more effective if a thrombin inhibitor (other than heparin) is administered simultaneously. Hirudin is a weak inhibitor of the thrombin-TM complex. New synthetic thrombin inhibitors may be better inhibitors of the thrombin-TM complex.

Recent data indicate that about 17% of the total u-PA antigen in plasma of healthy donors represents thrombin-cleaved scu-PA (Nauland et al. 1993), suggesting that inactivation of scu-PA happens in vivo. The physiological role of the inactivation of scu-PA by thrombin is possibly to protect the thrombus against premature lysis by captured and penetrating scu-PA. It is possible that reactivation of thrombin-cleaved scu-PA by still unknown enzymes may occur (Abercrombie et al. 1990, Nauland and Rijken 1993).

The activation of LMW scu-PA by plasmin is about three times slower than the activation of HMW scu-PA. This is reflected in a slower clot lysis by LMW scu-PA (chapter 7). The lower activity of LMW scu-PA is not an insuperable disadvantage: the difference with HMW scu-PA diminishes at higher scu-PA concentrations. In addition, more LMW scu-PA can be administered to obtain the same fibrinolytic effect.

LMW and HMW scu-PA have the advantage that the plasma levels can be easily controlled during thrombolytic therapy because both enzymes are cleared rapidly from the circulation by the liver. A disadvantage of such rapid clearance is that large amounts of scu-PA are needed. A specific receptor is probably responsible for the clearance (chapter 8), creating the opportunity for an inhibitor to develop which blocks the clearance. This clearance inhibitor could be administered instead of a thrombolytic agent, leading to a high endogenous scu-PA concentration for lysis of thrombi.

In summary, LMW scu-PA may be at least as suitable as HMW scu-PA for thrombolytic therapy because: 1) activation of plasminogen by both HMW and LMW tcu-PA is not stimulated by heparin, 2) the rate of inactivation by thrombin alone or in presence of thrombomodulin is equal, 3) the slightly slower activation rate with plasmin does not lead to a significantly slower clot lysis time at high scu-PA concentrations. 4) liver clearance rate and mechanism are equal.

9.3 Future investigations

Thrombomodulin is not only found on vascular endothelial cells, but also on extravascular cells (Boffa et al 1987, Imada et al. 1990, Wilhelm et al. 1992). Although the function there is unknown, TM may possibly be involved in the regulation of local u-PA activity via inactivation of scu-PA by thrombin. It would be interesting to investigate whether the thrombin-TM complex can inactivate receptor-bound scu-PA and if the complex is more active in the inactivation reaction when TM is bound to cells.

A major problem in thrombolytic therapy is the resistance to lysis of 25-30% of the thrombi in coronary arteries. It has been reported that thrombi wich are rich in platelets are more resistant to lysis. Therefore it is interesting to investigate whether there is a difference between the lysis rates of platelet-rich thrombi by LMW and HMW scu-PA.

In addition to the positive in vitro results, thrombolysis experiments with LMW scu-PA in animal models have shown that LMW scu-PA is an effective and selective thrombolytic drug in vivo (Spriggs et al. 1989, Stump et al. 1987). Clinical trials with LMW scu-PA should indicate the value of this agent.

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SUMMARY

Chapter 1 describes the physiological role, the mechanism and the therapeutic potencies of fibrinolysis. Fibrinolysis and coagulation are in balance in blood, preventing both the occlusion of blood vessels and bleeding. Fibrinolysis has a role not only in dissolving blood clots but also in a number of other processes, such as inflammation, cancer and tissue remodelling. In the process of fibrinolysis, plasmin is the active, fibrin-degrading enzyme, which is activated from its precursor plasminogen by t-PA (tissue-type plasminogen activator) or u-PA (urokinase-type plasminogen activator). Fibrinolytic therapy, often used in treatment of acute myocardial infarction, is based on the administration of plasminogen activators. This study investigates the fibrinolytic properties of low molecular weight (LMW) and high molecular weight (HMW) single-chain u-PA (scu-PA). LMW scu-PA lacks the amino-terminal part of HMW scu-PA which consists of a growth factor domain and a kringle domain.

Chapter 2 reviews the fibrinolytic properties of HMW scu-PA which were currently known at the beginning of the study. HMW scu-PA is an effective and specific fibrinolytic enzyme and is successfully used as a thrombolytic drug.

In chapter 3 we investigate the occurrence of different forms of u-PA in urine. Measurement by means of a bio-immunoassay shows that the urine samples contain 307 ± 189 ng/ml u-PA (mean of urine samples of ten healthy donors ± S.D.), of which 72 ± 15% is in the active two-chain form (tcu-PA) and 28 ± 15% in the single-chain form. No traces, or only slight traces, of u-PA-plasminogen activator inhibitor-3 (PAI-3) complexes are present in fresh human urine. Rapid complex formation occurs after desalting the urine and appears to be dependent on the presence of urinary sulphated glycosaminoglycans.

Chapter 4 further investigates the interaction of plasminogen activators and plasminogen with sulphated glycosaminoglycans (heparin). Heparin binding was observed with HMW u-PA and LMW u-PA, t-PA and plasminogen. The heparin binding of LMW u-PA is weaker than that of HMW u-PA. This is consistent with heparin-binding sites on both LMW scu-PA and the amino-terminal part of HMW u-PA. The activation of plasminogen by t-PA, HMW tcu-PA and LMW tcu-PA is stimulated 10- to 17-fold by heparin. The components probably form a cyclic ternary complex in which heparin acts as a template. The heparin stimulation of this reaction probably does not take place in vivo because physiological salt concentrations negate this stimulation. This salt sensitivity is mainly caused by the salt sensitive plasminogen-heparin interaction. The interactions between heparin and plasminogen activators are less salt sensitive and can occur under physiological conditions.

Thrombin-treated scu-PA is inactive and can hardly be activated by plasmin. Chapter 5 shows that inactivation by thrombin is seventy-fold accelerated by the thrombin receptor thrombomodulin (TM). The catalytic efficiency of this reaction (k_{cat}/K_m = 2.7 $\mu M^{-1}.s^{-1}$) is comparable with that of the activation of protein C by the thrombin-TM complex under comparable conditions (k_{cat}/K_m = 0.81 $\mu M^{-1}.s^{-1}$). LMW and HMW scu-PA are inactivated at the same rate by both free thrombin and the thrombin-TM complex. Conflicting results in this respect with others can be ascribed to the different LMW scu-PA preparations used (appendix to chapter 5). It can be concluded that the kringle and growth factor domains in HMW scu-PA have no role in the inactivation of scu-PA by the thrombin-TM complex. Chapter 6 investigates the role of the O-linked chondroitin sulphate on thrombomodulin in this reaction, by the use of two soluble recombinant TM mutants. A mutant which lacks the O-linked glycosaminoglycan (low-Mr rec-TM) has a lower affinity for thrombin than a fully glycosylated mutant (high-Mr rec-TM). The Kd for thrombin and low-Mr rec-TM and high-Mr rec-TM is 14 nM and 0.4 nM respectively. This confirms that the glycosaminoglycan moiety provides a secondary binding site for thrombin. It also appears that the thrombin-low-Mr rec-TM complex is much less active in the inactivation of scu-PA than the thrombin-high-Mr rec-TM complex. Thus, the O-linked chondroitin sulphate on TM plays a pivotal role in this reaction. It can be concluded from chapter 5 and 6 that thrombomodulin has not only anti-coagulant but also anti-fibrinolytic properties. The acceleration of the inactivation of scu-PA may imply a, as yet unidentified, mechanism for the regulation of local plasminogen activator activity on the cell surface.

In chapter 7, the fibrinolytic activities of LMW and HMW scu-PA are compared using a fibrin plate method, a purified fibrin clot lysis assay and a plasma clot lysis assay. In the three fibrinolytic assays, LMW scu-PA is 1 to 5 times less active than HMW scu-PA. The lower fibrinolytic activity can, at least partially, be explained by the observation that the activation of LMW scu-PA is about three times slower than the activation of HMW scu-PA.

The plasma clearance of the two scu-PA forms are investigated in chapter 8. Both LMW and HMW scu-PA are rapidly cleared from the circulation by the liver. LMW and HMW scu-PA are recognized by the same receptor on parenchymal liver cells, which is not the mannose or galactose receptor for glycoproteins or the known u-PA specific receptor which binds u-PA via its growth factor domain. It is suggested that this, as yet unknown, u-PA receptor in the liver is important for the regulation of the turnover of scu-PA.

Chapter 9 discusses the results of this study. It can be concluded that LMW scu-PA may be at least as suitable as HMW scu-PA for thrombolytic therapy.

SAMENVATTING

Hoofdstuk 1 beschrijft de fysiologische rol, het mechanisme en de therapeutische mogelijkheden van de fibrinolyse. Dit systeem is verantwoordelijk voor het afbreken van fibrine in bloedstolsels. In het bloed zijn de fibrinolyse en de stolling met elkaar in evenwicht om zowel afsluiting van bloedvaten als bloedingen te voorkomen. De fibrinolyse speelt niet alleen een rol in het oplossen van bloedstolsels, maar is ook betrokken bij een aantal andere processen zoals ontstekingen, groei van tumoren en herstel van weefsels. Plasmine is het actieve fibrine afbrekende enzym in de fibrinolyse en wordt gevormd uit het proenzym plasminogeen door t-PA (weefsel-type plasminogeen activator) of u-PA (urokinase-type plasminogeen activator). Fibrinolytische therapie, vaak toegepast bij de behandeling van het acute myocardinfarct, is gebaseerd op toediening van plasminogeen activatoren. In deze studie worden de fibrinolytische eigenschappen van laag moleculairgewicht (LMW) en hoog moleculairgewicht (HMW), plasmine activeerbaar één-ketenig u-PA (scu-PA) onderzocht. LMW scu-PA mist het aminoterminale deel van HMW scu-PA bestaande uit een groeifactor domein en een kringle domein.

Hoofdstuk 2 geeft een overzicht van de fibrinolytische eigenschappen van HMW en LMW scu-PA die al bekend waren voor de aanvang van deze studie. HMW scu-PA is een effectief en specifiek fibrinolytisch enzym en is succesvol gebruikt als trombolytisch middel voor fibrinolytische therapie.

In hoofdstuk 3 hebben wij het voorkomen van verschillende vormen van u-PA in urine onderzocht. Bepalingen met behulp van een bio-immunoassay laten zien dat urine monsters van tien gezonde donoren 307 ± 189 ng/ml u-PA bevatten (gemiddelde ± s.d.), waarvan 72 ± 15% in de actieve twee-ketenige vorm (tcu-PA) en 28 ± 15% in de één-ketenige vorm is. In verse urine is geen, of slechts een spoor van het u-PA-plasminogeen activator remmer-3 (PAI-3) complex aanwezig. Snelle complex vorming vindt plaats nadat urine ontzout is en blijkt afhankelijk te zijn van de aanwezigheid van gesulfateerde glycosaminoglycanen in urine.

In hoofdstuk 4 is de interactie tussen plasminogeen activatoren en lys-plasminogeen enerzijds en gesulfateerde glycosaminoglycanen (heparine) anderzijds verder onderzocht. Heparine binding werd gevonden voor HMW en LMW u-PA, t-PA en lys-plasminogeen. De heparine binding van LMW u-PA is zwakker dan die van HMW u-PA. Dit duidt op heparine bindingsplaatsen op zowel LMW u-PA als op het aminoterminale deel van HMW u-PA. De activering van lys-plasminogeen door zowel t-PA als HMW en LMW tcu-PA wordt door heparine 10 tot 17 maal gestimuleerd. Waarschijnlijk wordt een cyclisch ternair complex gevormd waarbij heparine als een mal fungeert. De heparine stimulatie vindt waarschijnlijk niet in vivo plaats omdat de stimulatie verhinderd wordt

door fysiologische zout concentraties. Deze zoutgevoeligheid wordt voornamelijk veroorzaakt door de zoutgevoelige interactie tussen heparine en lys-plasminogeen. De binding van de plasminogeen activatoren aan heparine is minder zoutgevoelig en kan wel een rol spelen onder fysiologische omstandigheden.

Trombine behandeld scu-PA is inactief en kan moeilijk door plasmine geactiveerd worden. Hoofdstuk 5 laat zien dat de inactivering door trombine met een factor 70 versneld wordt door de trombine receptor trombomoduline (TM). De catalytische efficiëntie van deze reactie (k_{cat}/K_m = 2,7 $\mu M^{-1}.s^{-1}$) is vergelijkbaar met die van de activatie van proteïne C door het trombine-TM complex onder vergelijkbare omstandigheden (k_{cat}/K_m = 0,81 $\mu M^{-1}.s^{-1}$). LMW en HMW scu-PA worden even snel geïnactiveerd door zowel vrij trombine als trombine in complex met TM. Tegenstrijdige resultaten van anderen konden worden toegeschreven aan verschillen in de gebruikte LMW scu-PA preparaten (appendix bij hoofdstuk 5). Geconcludeerd kan worden dat de kringle en groeifactor domeinen in HMW scu-PA geen rol spelen in de inactivatie van scu-PA door het trombine-TM complex. In hoofdstuk 6 is de rol van het O-gebonden chondroïtine sulfaat van TM in deze reactie onderzocht. Hierbij hebben we gebruik gemaakt van twee oplosbare recombinante TM mutanten. Een mutant waarbij de O-gebonden glycosaminoglycaan ontbreekt (laag-Mr rec-TM) heeft een lagere affiniteit voor trombine dan een volledig geglycosyleerde TM mutant (hoog-Mr rec-TM). De Kd voor trombine en laag- en hoog-Mr rec-TM is respectievelijk 14 en 0,4 nM. Dit bevestigt literatuurgegevens die aantonen dat de glycosaminoglycaan voorziet in een tweede bindingsplaats voor trombine. Vervolgens bleek dat het trombine-laag-Mr rec-TM complex veel minder actief is ten opzichte van scu-PA dan het trombine-hoog-Mr rec-TM complex. Het O-gebonden chondroïtine sulfaat van TM speelt dus een essentiële rol in de bestudeerde reactie. Uit hoofdstuk 5 en 6 kan de conclusie getrokken worden dat TM niet alleen anti-stollingseigenschappen heeft maar ook anti-fibrinolytische. De versnelling van de inactivering van scu-PA kan een mechanisme zijn waarmee plaatselijk op het celoppervlak de plasminogeen activator activiteit wordt gereguleerd.

In hoofdstuk 7 zijn de fibrinolytische eigenschappen van LMW en HMW scu-PA vergeleken met de fibrineplaat methode, met een gezuiverde clot lysis methode en met een plasma clot lysis assay. In de drie fibrinolytische assays is LMW scu-PA 1 tot 5 maal minder actief dan HMW scu-PA. De lagere fibrinolytische activiteit kan, tenminste gedeeltelijk, verklaard worden met de waarneming dat LMW scu-PA driemaal langzamer geactiveerd wordt door plasmine dan HMW scu-PA.

De plasma klaring van beide scu-PA vormen is onderzocht in hoofdstuk 8. Zowel LMW als HMW scu-PA worden snel door de lever uit de bloedsomloop geklaard. LMW en HMW scu-PA worden door dezelfde receptor op leverparenchymcellen herkend. Dit

is niet de mannose of galactose receptor voor glycoproteïnen of de bekende u-PA specifieke receptor die u-PA bindt via het groeifactor domein. Vermoedelijk is de nog onbekende u-PA receptor in de lever belangrijk voor de regulatie van de turnover van scu-PA.

In hoofdstuk 9 worden de resultaten van deze studie bediscussieerd. Geconcludeerd wordt dat LMW scu-PA minstens zo geschikt kan zijn als HMW scu-PA voor trombolytische therapie.

ABBREVIATIONS

PA	plasminogen activator
u-PA	urokinase-type plasminogen activator
t-PA	tissue-type plasminogen activator
scu-PA	single-chain urokinase-type plasminogen activator
Mr	relative molecular mass
PAI	plasminogen activator inhibitor
LMW	low molecular weight
HMW	high molecular weight
SDS	sodium dodecyl sulphate
PAGE	polyacrylamide gel electrophoresis
DFP	diisopropylfluorophosphate
SD	standard deviation
PCI	protein C inhibitor
BIA	bio-immunoassay
EDTA	ethylene-diamine tetra acetate
Tris	Tris-(hydroxymethyl)-aminomethane
IU	international units
U	units
hr	hour
min	minute
IgG	immunoglobulin G
kD	kiloDalton
S-2251	D-Val-Leu-Lys-paranitroanilide
S-2444	pyro-glu-gly-Arg-paranitroanilide
tcu-PA	two-chain urokinase-type plasminogen activator
rscu-PA	recombinant scu-PA
TM	thrombomodulin
rec-TM	recombinant thrombomodulin
CSGAG	chondroitin sulphate-like glycosaminoglycan
N	amino
C	carboxyl
KIU	kallikrein inhibitary units
BSA	bovine serum albumin
CU	casein units
HEPES	2-[4-(2-hydroxyethyl)-1-piperazinly]-ethane sulphonic acid

cpm	counts per minute
rpm	rotations per minute

CURRICULUM VITAE

Op 20 februari 1960 werd ik te Lisse geboren. Aldaar heb ik de lagere en middelbare school doorlopen. In 1978 behaalde ik mijn diploma Atheneum-B aan het Fioretti College en begon aan mijn studie biologie aan de Rijksuniversiteit te Leiden. Het kandidaatsexamen biologie, met als tweede hoofdvak scheikunde, werd in december 1981 afgelegd, het doctoraal examen in februari 1985. De doctoraal studie omvatte het hoofdvak plantenfysiologie/microbiologie (Botanisch laboratorium te Leiden) en de bijvakken moleculaire genetica (Biochemisch laboratorium te Leiden) en milieukunde (Centrum voor milieukunde te Leiden).

Van juni 1985 tot juli 1992 ben ik werkzaam geweest op het Gaubius Instituut TNO, nu deel van het IVVO-TNO Gaubius laboratorium te Leiden. De eerste drie jaar heb ik o.a. in het kader van mijn vervangende dienstplicht onder leiding van dr. J.H. Verheijen de struktuur-funktie relatie van t-PA onderzocht. Vanaf juni 1988 heb ik onder leiding van dr. D.C. Rijken onderzoek verricht naar de fibrinolytische eigenschappen van scu-PA, waarvan het resultaat in deze dissertatie is beschreven.