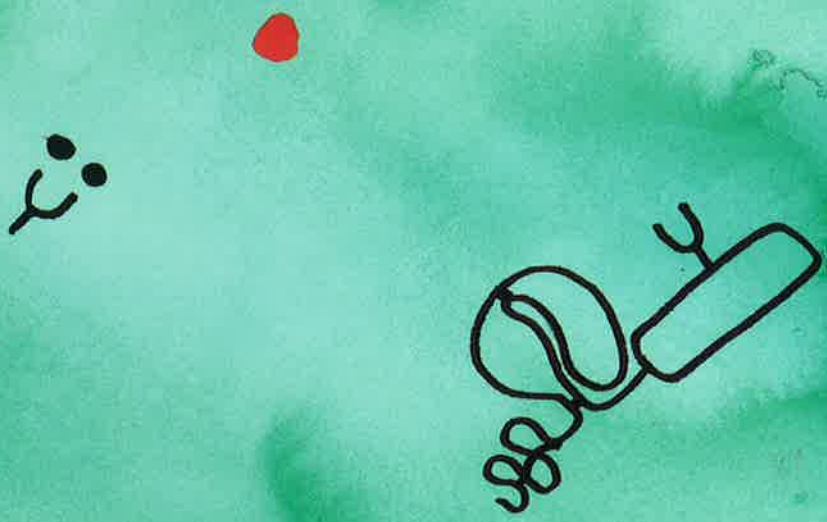
Identification of the receptors responsible for the clearance of urokinase-type plasminogen activator in rat liver



Marieke E. van der Kaaden

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Stellingen

behorende bij het proefschrift

Identification of the receptors responsible for the clearance of urokinase-type plasminogen activator in rat liver

1. Naast soortspecificiteit speelt bronspecificiteit ook een belangrijke rol in de herkenning van urokinase. *(Dit proefschrift)*

2. De conclusie van Collen *et al.* dat de herkenning van urokinase door de lever niet plaatsvindt via de suikerketen, alleen op basis van vergelijkbare turnover karakteristieken van een recombinant en een urinair urokinase preparaat, is niet terecht omdat uit de klaringssnelheid niet te concluderen valt welke receptoren verantwoordelijk zijn voor de klaring. *(Collen, D. et al., Thromb. Haemost. 1984, 52, 24-26).*

3. De aanwezigheid van sulfaat op de uiteinden van de complexe suiker van urinair u-PA verhindert de herkenning door de asialoglycoproteine receptor op de lever parenchymcellen. *(Dit proefschrift).*

4. Urinair tcu-PA kan beschouwd worden als een multireceptor ligand. *(Dit proefschrift).*

5. Complexvorming van urinair tcu-PA met zijn inhibitor PAI-1 leidt tot herkenning door LRP. *(Dit proefschrift).*

6. Het in studieboeken gebruikte model voor receptor-ligand herkenning als één slot en één sleutel dient na de ontdekking van de multiligand receptor LRP bijgesteld te worden.

7. De bewering van Narita *et al.* dat protamine sulfaat een inhibitor is voor heparan sulfaat proteoglycanen is alleen gebaseerd op het uitkomen van de verwachting dat de binding van TFPI door protamine sulfaat wordt gecompeteerd, terwijl dit niet gestaaft is door de cellen voor te behandelen met heparinase of door competitie met andere heparan sulfaat bindende liganden. *(Narita, M. et al., J. Biol. Chem. 1995, 270, 24800-24804).*

8. De mensheid heeft bescherming tegen infecties door de macrofaag scavenger receptor moeten bekopen met een verhoogde kans op atherosclerose. *(Suzuki, H. et al., Nature, 1997, 386, 292-296).*

9. Naast het uitrijden van de Elfstedentocht, is het uitzitten van deze tocht ook een hele prestatie.

10. Het ontstaan van een beperkt aantal farmaceutische multinationals komt de gezondheidszorg in het algemeen niet ten goede.

11. De steeds verder gaande automatisering in het wetenschappelijk onderzoek kan uiteindelijk leiden tot onderzoekers die als automaten werken.

12. Science kan niet zonder 'fantasy'.

Leiden, juni 1997 Marieke van der Kaaden

Identification of the receptors responsible for the clearance of urokinase-type plasminogen activator in rat liver

Proefschrift
ter verkrijging van de graad van Doctor
aan de Rijksuniversiteit te Leiden,
op gezag van de Rector Magnificus Dr. W.A. Wagenaar,
hoogleraar in de faculteit der Sociale Wetenschappen,
volgens besluit van het College van Dekanen
te verdedigen op dinsdag 24 juni 1997
te klokke 16.15 uur

door

Marie Elisabeth van der Kaaden
geboren te Berkel en Rodenrijs in 1967

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	Prof. dr. A. F. Cohen (Centre for Human Drug Research, Leiden)
	Prof. dr. R. M. Bertina (AZL, Leiden)

CIP-GEGEVENS KONINKLIJKE BIBLIOTHEEK, DEN HAAG

van der Kaaden, Marie Elisabeth
Identification of the receptors responsible for the clearance of urokinase-type plasminogen activator in rat liver / Marie Elisabeth van der Kaaden
Proefschrift Leiden - Met lit. opg.
ISBN 90-74538-23-1

The study presented in this thesis was performed at the Division of Biopharmaceutics of the Leiden/Amsterdam Center for Drug Research and the Gaubius Laboratory TNO-PG, Leiden and was supported by the Netherlands Heart Foundation (Grant 92.139).

Financial support by the Gaubius Laboratory TNO-PG in Leiden and by the Netherlands Heart Foundation of this thesis is gratefully acknowledged.

The printing of this thesis was further financially supported by the Dr. Ir. van der Laar Stichting.

It is the nature of an hypothesis, when once a man has conceived it, that it assimilates every thing to itself, as proper nourishment; and, from the first moment of you begetting it, it generally grows the stronger by every thing you see, hear, read, or understand.

Laurence Sterne (1713-68)
(Tristram Shandy (1759-67), bk. 4, ch. 5)

Aan mijn ouders
Voor Richard

Printed by Optima Druk, Molenaarsgraaf.

CONTENTS

Chapter 1

General Introduction

1.1. The fibrinolytic system

The fluidity of the circulating blood results from the balance between coagulation and fibrinolysis. The formation of a fibrin clot (coagulation) is necessary in order to prevent the leakage of blood when a blood vessel is damaged. Once the vascular damage has been repaired the fibrinolytic system is responsible for the dissolution of the fibrin clots. Insufficient coagulation or too much fibrinolytic activity results in sustained bleeding. Increased coagulation or defective fibrinolysis causes clotting (thrombosis), which leads to an interruption of the normal blood flow and the exclusion of organs from oxygen. This may lead to life threatening thrombotic or haemorrhagic events such as acute myocardial infarction, venous thrombosis or stroke.

An approach to treat thrombosis (thrombolytic therapy) is to dissolve the blood clot via infusion of plasminogen activators, which activate the fibrinolytic system in the blood. The fibrinolytic system, as depicted in figure 1, contains a proenzyme plasminogen which is converted to the key enzyme of this system, plasmin. Plasmin is a trypsin-like serine protease that digests fibrin into soluble degradation products.[1] Regulation of this process occurs at the level of the plasminogen activators by plasminogen activator inhibitors (mainly PAI-1) and at the level of plasmin by α_2-antiplasmin and α_2-macroglobulin. Plasmin degrades besides fibrin many other (plasma) proteins including the blood coagulation factors V and VIII, proteoglycans, fibronectin, laminin and latent proteases such as procollagenases[2]. As a result of degradation of components of the haemostatic system and vessel wall, excessive plasminogen activation may lead to bleeding complications.

Besides their role in the degradation of fibrin clots, plasminogen activators are also involved in ovulation[3,4], embryogenesis[5], angiogenesis[6], wound healing[7,8], neural development[9,10], inflammation, rheumatoid arthritis[11], tumor growth and metastasis.[12] In human plasma two plasminogen activators are present: tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA) in a concentration of about 0.1 nM. Both u-PA and t-PA are glycoproteins and are produced by vascular endothelial cells, u-PA is also synthesized by renal endothelial cells and a variety of malignant cell types. When produced in tumor

cell lines, the amount of u-PA is about 10 times higher than produced by normal cells.[13,14]

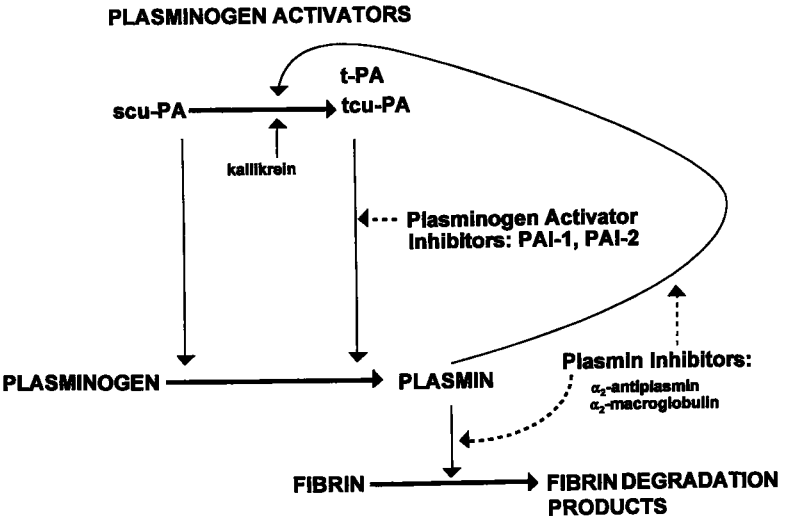

Figure 1: The fibrinolytic system. The bold arrows indicate conversion, the thin arrows indicate enzymatic activity, the striped arrows indicate inhibition. Abbreviations: scu-PA, single chain urokinase-type plasminogen activator; tcu-PA: two chain urokinase-type plasminogen activator; t-PA, tissue-type plasminogen activator; PAI, plasminogen activator inhibitor.

1.2. The urokinase-type plasminogen activator protein structure

Urokinase-type plasminogen activator (u-PA) is synthesized and secreted as a single-chain proenzyme with a molecular weight of approximately 55,000 by e.g. endothelial cells, kidney cells, fibroblasts and several tumor cell lines.[13-16] Single-chain u-PA (scu-PA) is composed of 411 amino acids which can be converted to two-chain u-PA by plasmin-catalyzed cleavage of the Lys_{158}-Ile_{159} peptide bond.[17] Urokinase is a mosaic protein consisting of 4 domains on the base of the primary structure: 1) epidermal growth factor domain, 2) kringle domain, 3) the connecting peptide domain (together called the A- or light chain), and 4) the proteolytic domain (called the B- or heavy chain) (figure 2).

The epidermal- and kringle domains have significant homology with domains found in other proteins and are probably evolutionary related.[18] Residues 5-45 are called the epidermal growth factor (EGF) domain, because of their homology with

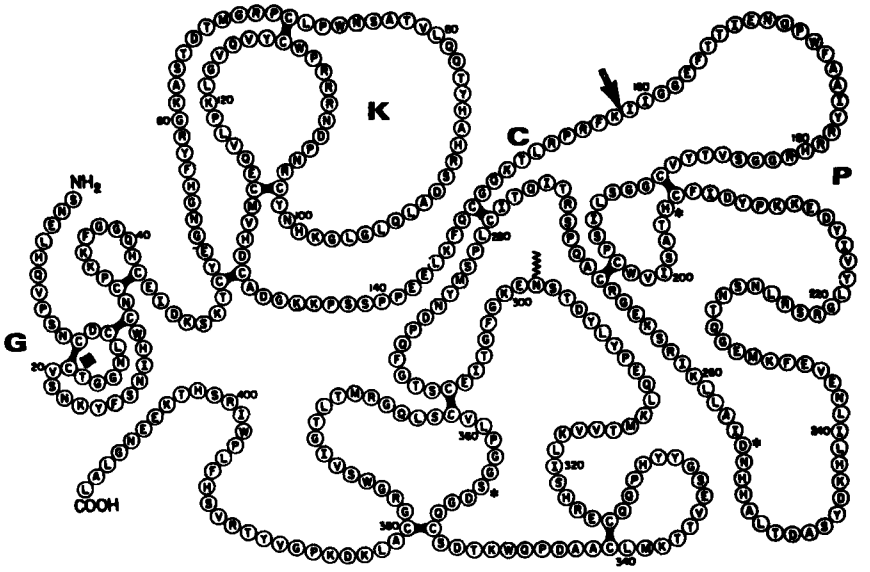

Figure 2: Schematic structure of human scu-PA. The arrow indicates the cleavage of the Lys_{158}-Ile_{159} bond resulting in active tcu-PA. The cysteine residues have been joined by a black bar representing a disulphide bond. The zigzag line indicates the complex carbohydrate side chain at Asn_{302}, ♦ indicates the O-linked α-fucose at Thr_{18}. * indicates the three active site amino acid residues. G, growth factor domain; K, kringle domain; C, connecting peptide domain; P, proteolytic domain (figure obtained and modified from reference 31).

the epidermal growth factor.[19,20] The kringle domain (residue 46-134) is homologous to the kringle domains in t-PA, plasminogen and prothrombin.[21] It may be involved in heparin binding via $Arg_{57\text{-}60}$.[22,23] The proteolytic domain (residue 159-411) contains the active centre formed by the amino acids His_{204}, Asp_{225} and Ser_{356} that converts plasminogen to plasmin.[21,24] The growth factor and kringle domain are independently folded and do not interact with each other or with the protease domain.[25-27] The protease domain is built up of two domains that behave independently at low pH but form a single unit at neutral pH.[27,28] Due to the presence of the connecting peptide (residue 135-158) there is a great motional freedom between the kringle and protease domain.[29] Hamelin *et al.*[30] have shown that the cysteine residues forming disulfide bonds in the EGF- and kringle domain are not necessary but those in the proteolytic domain are essential for maintaining amidolytic and fibrinolytic activity of high molecular weight single-chain u-PA (HMW-scu-PA).

Several molecular forms of u-PA have been described. The first originates from

the cleavage of HMW-scu-PA (= 55 kDa) between Lys_{158} and Ile_{159} by plasmin to form the active high molecular weight two-chain u-PA (HMW-tcu-PA). The two peptides are held together by a disulfide bond between Cys_{148} and Cys_{279}.[31] During the conversion of scu-PA to active tcu-PA the conformation of the molecule is changed, resulting in the exhibition of the active site.[32] Further proteolytic digestion by plasmin between Lys_{135}-Lys_{136} results in the Amino Terminal Fragment (ATF, 22 kDa) of the protein (residue 1-135) and Low Molecular Weight u-PA (LMW-u-PA, 33 kDa, residue 136-411).[33] Scu-PA obtained from cultured human kidney cells may be cleaved between Glu_{143} and Leu_{144} resulting in a LMW-scu-PA form of 32 kDa.[34] Marcotte *et al.*[35,36] identified the enzyme responsible for the cleavage of the Glu_{143}-Leu_{144} bond as Pump-1 (putative metalloproteinase). Another molecular form is the result of thrombin mediated cleavage of the Arg_{156}-Phe_{157} bond.[37,38] This reaction is accelerated by the endothelial receptor thrombomodulin.[39] The HMW-tcu-PA found in urine has been shown to have Phe_{157} as the carboxyl-terminal residue in ATF instead of Lys_{158} probably due to the action of a carboxypeptidase.[40] Scu-PA can be activated by plasmin[41,42], trypsin[13,16], plasma kallikrein[37], and several other enzymes. It is still unknown whether these reactions occur *in vivo*, but both kallikrein and plasmin are likely activators.

u-PA has a complex-type carbohydrate side chain at Asn_{302}[43] and an O-linked α-fucose at Thr_{18}[43,44] The fucose may be involved in mitogenesis and the stimulation of tumor cell proliferation by u-PA.[45,46] Monosaccharide analysis of the complex-type carbohydrate chain of urinary u-PA revealed the occurrence of fucose, galactose, mannose, GlcNAc, Neu5Ac in the molar ratio of 2.0, 1.4, 3.0, 3.2, 3.4, 1.1, respectively.[47] It was shown by Bergwerff *et al.*[48] that the isolated N-glycans are a collection of di- and tri-antennary structures that contain predominantly GalNAcß1-4GlcNAcß terminal elements.[47] The GalNAc residue may be sulfated at C4, or to a lesser extent may bear N-acetylneuramic acid at C6; alternatively the GlcNAc residue can be fucosylated at C3. The major component, which accounts for more than 30 % of the total oligosaccharide pool, consists of an α1-6-fucosylated diantennary carbohydrate chain with (SO_4)-4GalNAcß1-4GlcNAcß1-2 antennae. The observed large heterogeneity of u-PA purified from a pool of human urine from numerous donors does not necessarily mean that u-PA from a single individual will exhibit the same variation of N-glycans. Glycosylated u-PA from other sources, which does not contain the GalNAcß1-4GlcNAcß1-2 terminal elements, may or may not be capped at each antennary branch with a single sialic acid residue.[49] Furthermore, urokinase is phosphorylated in Ser, Tyr and in Thr.[50-52] Phosphorylation of u-PA decreases its affinity for PAI-1 which implies an increased enzyme activity.[53,54]

u-PA knock out mice have been generated by Carmeliet *et al.*[55] The mice have a normal development, are fertile and have a normal life span. Occasionally they develop spontaneously minor fibrin deposits in liver and intestine and excessive

fibrin deposits in chronic nonhealing skin ulcerations.[55,56] The lysis rate of an experimental clot injected in the jugular vein in knock out mice is comparable to that in wild-type mice (≈ 67 % lysis over 16 hours).[57]

1.3. Thrombolytic therapy

Early attempts to treat thromboembolic disorders focused on the prevention of thrombus formation and the prevention of extension of an existing thrombus. Thrombolytic therapy for these conditions included intravenous and oral administration of anticoagulants (such as heparin) and antiplatelet agents (such as aspirin).[58] Because anticoagulants and antiplatelet agents are unable to dissolve fibrin clots once they have been formed, agents that could lyse fibrin clots directly were sought.

The ideal thrombolytic agent should be thrombus selective, non-immunogenic, quickly acting and highly effective. The first agents used were streptokinase and HMW-tcu-PA. Although it was shown that HMW-tcu-PA and streptokinase are effective thrombolytic agents, they cause extensive systemic fibrinogen breakdown[59] and lead to bleeding complications. Both drugs lack specific affinity for fibrin and will therefore activate both circulating and fibrin-bound plasminogen. The abundantly produced plasmin is rapidly inactivated by α_2-antiplasmin (the half-life of plasmin is approximately 0.1 sec), leading to an exhaustion of the amount of α_2-antiplasmin in the blood.[60] This causes an increased level of proteolytic active plasmin in the circulation, which will degrade several plasma proteins (e.g. fibrinogen, factor V and VIII). Excessive lowering of the concentrations of these blood components combined with the inhibitory effect of some fibrin degradation products on platelet aggregation and fibrin polymerization may contribute to the bleedings.[58,61]

The "second generation of thrombolytic agents" are: recombinant tissue-type plasminogen activator (rt-PA), single-chain u-PA (scu-PA) and anisoylated plasminogen streptokinase activator complex (APSAC) a complex of streptokinase and plasminogen.[1,61,62] A disadvantage of the use of streptokinase and APSAC is that when injected in the circulation antibodies are generated and therefore a second treatment with this reagents will lead to an unwanted immuneresponse. Recombinant t-PA is a more effective and fibrin specific thrombolytic agent than streptokinase.

Goldhaber *et al.*[63] showed that in case of pulmonary embolism, administration of a concentrated dose of u-PA in a short period of time (10 min bolus, followed by 110 min infusion) gives similar efficacy and safety as t-PA (100 mg/2 hour). Also the outcome of the SESAM Trial[64] showed that a rapid administration of rscu-PA (80 mg/1 hour) or rt-PA (100 mg/3 hours) in patients with myocardial infarction resulted in high early patency rates for both drugs, with a trend towards earlier patency in the scu-PA group.

Because of its higher fibrin-selectivity[16,65,66], its affinity for plasminogen[67-69], the better stability in plasma[70] and the inability to form u-PA:PAI-1 complexes[70] HMW-scu-PA is a more suitable candidate for thrombolytic therapy than HMW-tcu-PA. Therefore HMW-scu-PA is now investigated as a relatively fibrin-specific thrombolytic agent in patients with acute myocardial infarction.[71-74] In plasma, in the absence of fibrin, HMW-scu-PA is stable and does not activate plasminogen.[70] However, in the presence of a fibrin clot HMW-scu-PA induces fibrin-specific clot lysis.[66,75] Since HMW-scu-PA has no direct affinity for fibrin[76,77], its clot specific action cannot be explained via binding to fibrin.[70,77] Fleury *et al.*[78] showed that fibrin may serve as a contact catalyst able to provide a reaction site for the intrinsic proteolytic activity of HMW-scu-PA (1 % of that of HMW-tcu-PA).[79] They showed that plasminogen is associated to the carboxyl-terminal lysine residues of degraded fibrin. HMW-scu-PA binds to this plasminogen and converts it into plasmin after which HMW-scu-PA separates from the fibrin-bound plasmin or can be activated to tcu-PA by the fibrin-bound plasmin. This results in the generation of fibrin-bound plasmin and explains the fibrin selectivity of HMW-scu-PA. No conversion of plasminogen to plasmin by HMW-scu-PA occurs on an intact fibrin surface. Since carboxy-terminal lysine residues are most likely the result of plasmin activity, the plasminogen activation by HMW-scu-PA will only take place after plasmin degradation. It is therefore likely that the above described pathway of plasmin activation mainly plays a role in the prolongation phase of fibrinolysis and that for the initiation of fibrinolysis other pathways exist.

A large clinical trial, the PRIMI Trial, showed that upon intravenous injection of *E.coli* produced recombinant scu-PA (=rscu-PA, 20 mg bolus followed by a 60 mg infusion over 1 hour) or streptokinase (=SK, $1.5*10^6$ IU/60 min) in patients with acute myocardial infarction, rscu-PA acted faster than SK in clot lysis (patency rate at 60 min of 71.8 % and 48 % for rscu-PA or SK respectively).[71] Furthermore, the use of rscu-PA led to a lower rate of early reocclusion of the vessels, earlier reperfusion, less disturbance of haemostasis and fewer bleeding complications than the use of SK. Pharmacokinetic and pharmacodynamic studies of rscu-PA in healthy volunteers[80] and patients[81] showed that about 25 % activation to tcu-PA occurs. The generated tcu-PA may contribute to the thrombolytic efficacy when generated at the clot surface or in the circulation. Also for the treatment of deep venous thrombosis u-PA is a promising thrombolytic agent.[82,83]

Recently Samama and Acar[84] stated that there is a "golden first hour" in the treatment of acute myocardial infarction. During the first hour of treatment following the onset of symptoms the reduction in mortality is about 50 % after which the beneficial effect of thrombolytic therapy declines. For all the plasminogen activators, aspirin administration virtually doubles the benefit.[85] Concomitant administration of heparin with plasminogen activators during thrombolytic therapy is used to prevent new fibrin formation and rethrombosis.[86] Heparin is not likely to significantly accelerate the plasminogen activation reaction

in vivo.[87] Probably the interaction of plasminogen activators with heparin and related glycosaminoglycans may be important to elevate the concentrations of plasminogen activators on the cell surfaces or to modulate the reactivity of the plasminogen activators in various physiological processes. For example heparin stimulates the plasmin-mediated conversion of scu-PA to tcu-PA.[88]

In conclusion, an optimal thrombolytic therapy using scu-PA should rapidly start after the infarction, and infusion of scu-PA should take place in a short period of time (1-2 hours). Furthermore since scu-PA is not able to discriminate between fibrin in a thrombus and in a haemostatic plug, bleeding complications can occur. Therefore, thrombus specific targeting for instance by coupling u-PA to a thrombus specific antibody may be an additional advantageous approach.

1.4. Clearance of u-PA

The clearance of u-PA is described in various species, like rabbits, squirrel monkeys, dogs, mice, rats and men.[89-92] Injection of HMW-scu-PA, HMW-tcu-PA or LMW-scu-PA led to a rapid disappearance from the circulation with an average half-life ranging from 3 minutes for mice and rats to 8 minutes for men. The plasma clearance of LMW-scu-PA is slightly slower than that of HMW-scu-PA.[93] The main organ for the uptake of u-PA appears to be the liver.[90,94] Henkin *et al.*[95] determined in rabbits that the sialic acid content in glycosylated HMW-scu-PA influences the plasma clearance. They have described that recombinant HMW-scu-PA produced by CHO cells, containing 2.5-3.0 molecules sialic acid per molecule was cleared more slowly than less sialyated HMW-scu-PA obtained from SP2/0 or human kidney cell cultures ($\leq$ 2.5 molecules sialic acid per molecule HMW-scu-PA).

Gurewich *et al.*[96,97] showed that 20 % of the endogenous HMW-scu-PA in blood is associated with a ≈70 kDa protein[97,98] on platelets. Only HMW-scu-PA was associated with the platelets and recognition occurred via the amino terminal fragment of HMW-scu-PA. The recognition was not mediated via carbohydrate chains on HMW-scu-PA, since both native glycosylated and recombinant non-glycosylated HMW-scu-PA associated with the platelets. The physiological role of platelet-association of HMW-scu-PA is not known yet, but one can imagine that association of a part of HMW-scu-PA to circulating platelets extends the half-life of u-PA. This may contribute to the exceptionally low rate of rethrombosis at 24 h after infusion of HMW-scu-PA in coronary thrombolysis.[71,99] Furthermore, it has been shown that plasminogen, the u-PA substrate, and kallikrein, an activator of scu-PA, also bind to the platelet surface.[100] Therefore platelets localize all the components of u-PA mediated plasminogen activation on their surface and may play in this way a role in fibrinolysis.

1.5. The u-PA Receptor (uPAR)

In 1985 Vassalli *et al.*[101] reported that scu-PA and tcu-PA bind to a specific cell surface receptor (uPAR) on human blood monocytes and the monocyte-like U937 cell line, with high affinity ($K_d \approx 10^{-9}$-10^{-10} M, depending on the cell type). Many other authors have now demonstrated the presence of uPAR on a variety of cell lines of neoplastic and non-neoplastic origin.[102-104] Amongst the latter are polymorphonuclear leukocytes[105], lymphocytes[106], natural killer cells[107], vascular endothelial cells[24,108], smooth muscle cells[109] and keratinocytes.[110,111] The biochemical characteristics of uPAR on the different cells types appear very similar, however with differences in the pattern or extent of glycosylation.

1.5.1 Structure of uPAR

The human uPAR is a glycoprotein of Mr 55,000-60,000[104,112] which is anchored to the cell surface by a covalent linkage of the carboxy-terminal amino acid of the protein to a glycosylated form of the phospholipid phosphatidyl-inositol, resulting in a complex glycolipid anchor termed "glycosyl-phosphatidyl inositol anchor" (GPI-anchor).[113] The uPAR can be released from the cell surface by a phosphatidyl inositol-specific phospholipase C (PI-PLC).[114] The GPI moiety is attached to uPAR during posttranslational events which include a cleavage in the carboxyl-terminal part of uPAR so that GPI becomes attached to the novel carboxyl-terminus Gly_{283}.[114,115]

The amino acid sequence of human uPAR contains an internal triple repetition of the spacing of the cysteines. This cysteine repetition is also present in a few other GPI-linked proteins which form the Ly-6 family, suggesting that uPAR would be formed as a three domain structure.[113,116] The motives are coded: domain I: residue 1-92, domain II: 93-191, and domain III: 192-283 (figure 3). The 85 amino terminal residues can be cleaved off by chymotrypsin and contain the major ligand-binding affinity.[116] Studies with domain I of uPAR showed a 1500-fold decrease in u-PA binding affinity compared to the intact uPAR molecule. This demonstrates that domain I does not contain all the determinants necessary for u-PA binding.[117] Although domain II and III are devoid of binding activity it was determined that domain II or III are required to stabilize the active conformation of domain I.[118,119]

Different forms of uPAR and its mRNA have been identified[120,121] including proteolytically cleaved forms that lack the ligand binding domain.[122] Proteolysis can be generated by urokinase and/or plasmin. Soluble forms of uPAR lacking at least part of the GPI-anchor have also been described.[123,124]

uPAR contains 5 potential N-glycosylation sites[125] of which Asn_{52} in domain I and at least one of the other glycosylation sites are glycosylated.[116,126] Mutation of Asn_{52} to Gln_{52} results in a uPAR with a 5-6-fold lower affinity for u-PA.[126] Differences in glycosylation have been observed between various cell types that express u-PAR.[127] This heterogenous glycosylation pattern of uPAR may be

responsible for differences in its affinity for u-PA.[128]

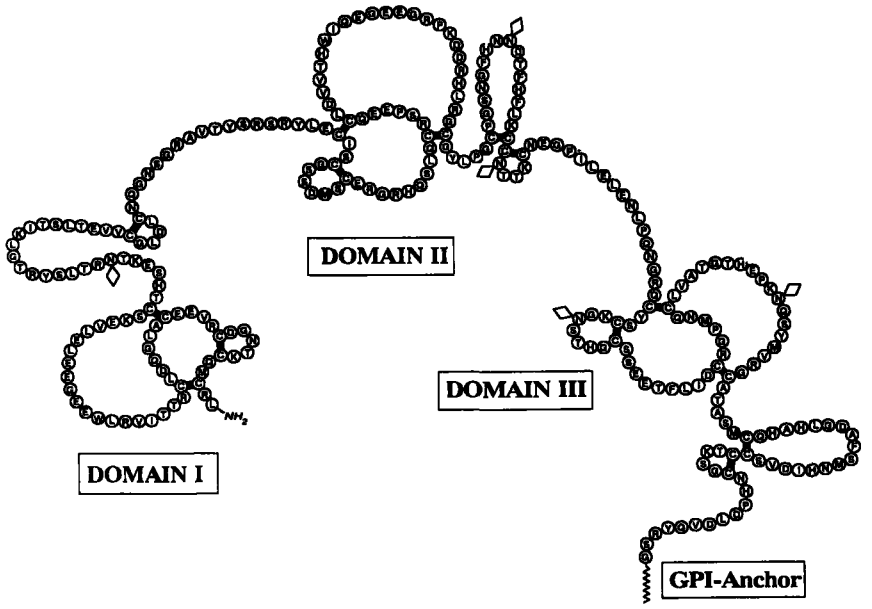

Figure 3: Schematic structure of human uPAR. The three-domain structure of uPAR is shown. The cysteine residues have been joined by a black bar representing a disulphide bond. ◊ represent the potential attachment sites for N-linked carbohydrates (Figure from reference 129).

1.5.2. Characteristics of the u-PA:uPAR binding

uPAR recognizes scu-PA, tcu-PA, tcu-PA:PAI-1 and diisopropylfluorophosphate (DFP)-treated tcu-PA[43,101,130] with high affinity (0.1-1.0 nM).[101] u-PA binds via its ATF[102] to uPAR and Appella *et al.*[131] showed by constructing synthetic peptides of u-PA that the residues 4-43 contain the receptor recognition sequence. The residues 20-30 are critical for binding of u-PA to uPAR, residues 4-19 and 33-43 maintain the proper conformation of the binding region. Magdolen *et al.*[132] showed that within the receptor binding sequence (residue 20-30) the aromatic or lipophilic side chains of Asn_{22}, Tyr_{24}, Phe_{25}, Ile_{28} and Trp_{30} which all point to one side, are elements for the specific hydrophobic interactions of u-PA within the binding pocket of uPAR. The substitution of the afore mentioned amino acids by

Ala reduced the interaction of u-PA with uPAR dramatically. The expression of uPAR varies from cell type to cell type[101] and is regulated by a variety of growth factors, cytokines and hormones.[126,133,134]

A cleaved form of uPAR has been described on tumor cells by Hoyer-Hansen *et al.*[135] They have shown that u-PA is involved in the cleavage of uPAR on tumor cells both *in vitro* and *in vivo*. Domain I is cleaved off, while domain II and III remain anchored to the cell surface. This cleavage is inhibited by antibodies against u-PA, indicating that the cleavage is catalyzed by either u-PA or plasmin generated by u-PA.[136] The cleavage of domain I from uPAR abolishes the binding of u-PA to the tumor cells and therefore reduces plasminogen activation at the cell surface. Cleavage of uPAR may therefore be a negative regulation mechanism which limits the extent of cell-surface plasminogen activation. It is also an alternative pathway to the PAI-1-mediated inhibition of u-PA activity.

The uPAR also plays a role in tumor metastasis[137], cellular invasion[138,139], cell migration[140] and chemotaxis[141,142] by localizing u-PA at the cell surface. During the process of tumor cell invasion and metastasis cells have to leave the primary site of the tumor, migrate through the extracellular matrix of the surrounding tissue, penetrate the basement membrane of a blood vessel and extravasate into a target organ. These processes require tumor cells to secrete a number of different proteases, including u-PA.[2] Many invasive tumor cells express both HMW-scu-PA and uPAR. The secreted HMW-scu-PA binds to uPAR, and after activation to HMW-tcu-PA, plasmin can be generated that in turn digests extracellular proteins in the surrounding tissues. High levels of uPAR in breast and lung cancer tissue are, like high u-PA levels, associated with poor prognosis. Interference with the u-PA:uPAR interaction represents a promising new approach for anti-invasive therapy.

uPAR-deficient mice were generated by Bugge *et al.*[143] with no phenotypic abnormalities, indicating that the uPAR mediated processes are not essential for fertility, development or haemostasis. This can be explained by the presence of other binding sites for u-PA or the presence of other proteases, such as metallo-, serine- and thiol-proteases, that appear to be necessary for the modification and degradation of complex extracellular matrices with diverse protein composition. If the proteases share a partial functional overlap then it follows that the loss of any one component would not necessarily compromise overall extracellular matrix remodelling or turnover.

1.5.3. Species specificity

Although the receptor-binding regions of human- (huPAR) and mouse uPAR (muPAR) share 62 % amino acid sequence homology[144] and conservation of all cysteine residues[145] a remarkable species specificity is observed. Mouse u-PA binds to mouse uPAR, but hardly to human uPAR, whereas human uPA binds only to

human uPAR.[146] Southern blotting experiments with genomic DNA showed that a huPAR probe hybridized only with human, monkey and bovine uPAR but not with rat, mouse, dog and rabbit DNA.[147] Pollanen *et al.*[148] identified within domain I two regions critically involved in the u-PA binding. The first region (residues 1-13) contains 6 non-conserved residues between mouse and human uPAR of which residue 2, 7, 8, 10 are important for the binding specificity. After introduction of the murine residues into huPAR, huPAR recognized murine u-PA, whereas introduction of the human residues in muPAR resulted in the recognition of human u-PA by muPAR. The second region (residue 47-53) important for u-PA binding contains highly charged residues and is conserved between human and mouse uPAR and has an overall structural function in the binding. Also within the uPAR-binding region of u-PA (residue 12-32) 36 % of the amino acid residues are different between mouse and human u-PA.[146] Therefore, in conclusion differences in both u-PA and u-PAR recognizing sequences may contribute to the observed species specificity.

1.6. The LDL-Receptor family

The LDL receptor family is a family of receptors that include the Low Density Lipoprotein Receptor (LDLr)[149], the Low Density Lipoprotein Receptor-related Protein (LRP)[150], the Very-Low Density Lipoprotein Receptor 1 and 2 (VLDLr-1, VLDLr-2)[151,152] and epithelial glycoprotein 330 (gp330)[153] (figure 4). A molecule closely related to LRP has been identified in *C.elegans.*[154] The chicken and *Drosophila* vitellogenin receptor also belong to the LDL receptor family.[155,156]

The member of this family, that was first identified, was the LDLr, which mediates the cellular uptake of apolipoprotein B (apoB) and/or apoE containing lipoproteins.[157] The extracellular portion of the LDLr consists of an O-linked sugar domain and two clusters of cysteine-rich repeats. The first cluster shows homology with the epidermal growth factor precursor and contains three epidermal growth factor like repeats (EGF repeat) that are separated by five copies of a repeat, containing a common tetrapeptide, tyrosine-tryptophan-threonine-aspartic acid (EGF-precursor homologous domains). The second cluster contains seven complement-like repeats, which are responsible for ligand binding (ligand binding repeat). The cytoplasmic tail contains an NPXY sequence, an internalization signal required for clustering of the receptor in clathrin-coated pits.

The second family member, LRP is much larger than the LDLr (4525 vs. 839 residues).[157] The cytoplasmic domain of LRP contains two copies of the NPXY coated pit internalization signals and the O-linked sugar domain of the LDLr is replaced by six growth factor-like repeats. LRP contains 22 EGF repeats and 31 ligand binding repeats arranged into four clusters containing 2, 8, 10 and 11 complement-type repeats per cluster (CRP)[158], respectively. Together the ligand binding repeats contain a net negative charge of 158, suggesting that the receptor

is capable of binding positively charged ligands. Sequence analysis showed that LRP is identical to the α_2-macroglobulin receptor (α_2-MR).[150,159,160] LRP is widely distributed in numerous cell types exposed to plasma or interstitial fluid. High levels of LRP expression were detected in parenchymal liver cells, placenta and monocytes/macrophages.[150,161-163]

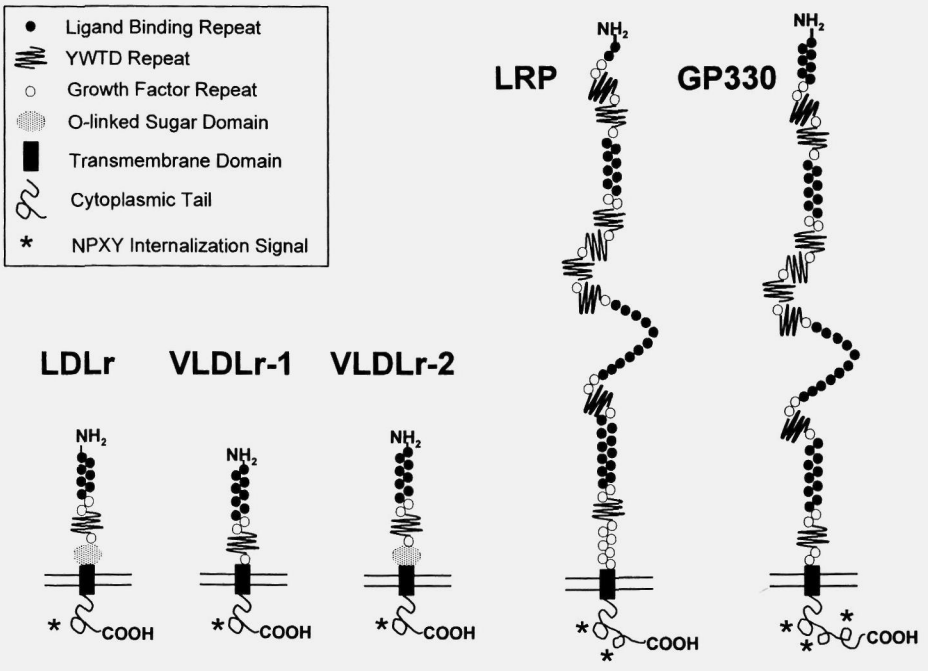

Figure 4: The LDL receptor family. All members of the LDL receptor family consist of the same structural motifs that are described in the box.

A third member of the LDL receptor family is gp330. The overall size and domain organisation of gp330 is the same as for LRP.[164] The cytoplasmic tail contains three NPXY sequences. Except for retinal and ciliary epithelial cells, gp330 and LRP are expressed in different tissues.[163] Gp330 was found in a restricted group of epithelial cells, including renal proximal tubule epithelium, glomerular epithelial cells, podocytes, type II pneumocytes, thyroid, epididymis, lining of the uterus, retina and the yolk sac.[162,163]

The fourth member of the LDL receptor family is the VLDLr. The VLDLr is

highly abundant in the heart, (skeletal) muscle and adipose tissue but not present in the liver.[151] Furthermore, VLDLr is present in the brain, spleen, lung, kidney, adrenal, testis, small intestine[151], human vein endothelial cells and human aortic smooth muscle cells.[165] Two types of VLDLr's are identified: VLDLr-1 and VLDLr-2. They differ only in the presence (VLDLr-2) or absence (VLDLr-1) of an O-linked sugar domain (figure 4).

The most recently described members of the LDL receptor family are predominantly present in the brain and probably have a brain-specific function. A first new receptor of the family is besides in brain also present on human umbilical vein endothelial cells, microvascular endothelial cells and human arterial smooth muscle cells.[166] This protein of 837 amino acids shows 67 % sequence homology to the LDLr, and 57 % to the VLDLr.

A second new family member is named the apolipoprotein E receptor 2 (apoER2) and consists of five functional domains resembling the LDLr and VLDLr for 50 %.[167] It is a protein of 922 amino acids which recognizes apoE enriched ß-VLDL with high affinity. LDL is recognized with much lower affinity.

A third new member that is abundantly present in the brain is the LR11 receptor.[168] This receptor of 2213 amino acids that is also present in liver, adrenal glands and testis, binds apoE containing lipoproteins. It is an unusually complex member since it is built up of a cluster of 11 LDLr ligand binding repeats, 5 YWTD binding repeats, a domain that is structurally related to a neural cell adhesion molecule module, and a domain with similarities to a yeast receptor for vacuolar protein sorting.

Finally a fourth recently described brain specific member is termed LR8B.[169] This highly glycosylated protein of 130 kDa discovered in chicken and mice, shows great homology with the ligand binding domains of LR8 in chicken and the VLDLr in mammals. Between mouse and chicken LR8B a 73 % overall identity is observed. This receptor is not present in the liver, muscles, lung, heart, kidney and ovary.

1.6.1. The Low Density Lipoprotein Receptor Related Protein (LRP): biosynthesis

LRP is synthesized in the endoplasmic reticulum as a single chain precursor, of 4525 residues with a molecular mass of 600 kDa.[170] After it reaches the Golgi complex, the protein is cleaved by the resident endoproteinase furin between residue 3924 and 3925 to generate two subunits, one of 515 kDa (α-chain, extra cellular) and one of 85 kDa (ß-chain, membrane spanning).[170,171] Both subunits remain non-covalently associated to each other. During its passage through the cell the protein is modified by N-linked glycosylation.[170] Jensen *et al.*[172] showed that a wide variety of glycans contribute for 18 % and 25 % to the size of the α- and ß-chain, respectively. LRP plays an essential role during embryonic development since LRP knock-out mice are not viable.[173]

1.6.2. The Receptor-Associated Protein (RAP): an antagonist of the LDL-receptor family

During purification of LRP by affinity chromatography, a 323 amino acids long Receptor-Associated Protein (RAP) was identified.[160,174] This 39 kDa glycoprotein[172] binds with high affinity to LRP (K_d = 4 nM) and antagonizes the binding of all known ligands to this receptor.[175-177] RAP binds to at least two equivalent binding sites on LRP.[175] RAP also binds with high affinity to the VLDLr (K_d = 0.7 nM)[178] and to gp330 (K_d = 8 nM)[176], but with low affinity to the LDLr (K_d = 500 nM).[179] RAP is synthesized in the endoplasmic reticulum but is not secreted by the cells[180], since it is mainly associated to the endoplasmic reticulum membrane.[177,181]

RAP is the homologue of a mouse protein termed heparin-binding protein 44.[180] Warshawsky *et al.*[182] showed by constructing mutants of RAP that a heparin binding site within RAP is located in the carboxy terminus (amino acids 115-319). Orlando *et al.*[183] showed that RAP possesses the heparin binding site between amino acids 261 and 323. It was shown by Ji *et al.*[184] that RAP binds to heparan sulfate proteoglycans on CHO cells. Removal of the heparan sulfate proteoglycans with heparinase treatment reduced the binding of ^{125}I-RAP by 40 %. Mutant CHO cells lacking heparan sulfate proteoglycans bound only 50 % of the amount ^{125}I-RAP compared to the wild-type CHO cells. The binding of RAP to heparan sulfate proteoglycans may be dependent on the cell type, since different cells may display specific types of heparan sulfate proteoglycans on their surface. On human fibroblasts for instance, RAP binds to LRP and to a high capacity, low affinity site.[185] The binding of ^{125}I-RAP was abolished by heparin. However no significant binding to heparan sulfates was detected, since heparinase or sodium chlorate treatment did not decrease the binding of RAP to the fibroblasts.

RAP functions as an intracellular molecular chaperon for LRP and regulates its ligand binding activity along the secretory pathway.[186] RAP associates with LRP early in the secretory pathway thereby maintaining LRP in a non-ligand binding state. When RAP dissociates from LRP late in the secretory pathway due to a lower pH, the ligand binding activity of LRP increases. By maintaining LRP in this inactive state it is prevented that other ligands, which may travel by the same secretory pathway as LRP (e.g. t-PA, u-PA, apoE), interact with the receptor before it reaches the cell surface. The chaperon role of RAP was further established by a study of Willnow *et al.*[187,188] who showed by the generation of RAP-deficient mice that the LRP expression in liver and brain is reduced by 75 % while the mRNA concentration was not affected. The residual LRP within the RAP deficient liver cells appeared to be in the unspliced 600 kDa precursor form instead of the mature 85 and 515 kDa form. Furthermore LRP forms aggregates in the endoplasmatic reticulum of the knock out liver cells. The LRP expression was restored to normal levels by the transfer of RAP cDNA in the knock out liver.[188]

A second intracellular role for RAP is recently shown by Bu *et al.*[189] They showed that RAP plays a role in the folding of LRP by interacting with the second,

third, and fourth cluster of complement-type repeats (CRD-II, III, IV) of LRP. These interactions are necessary to obtain the proper folding of the protein by ensuring the formation of intradomain disulfide bridges and not intermolecular or interdomain disulfide bridges. Cellular expression of LRP in the absence of RAP resulted in the formation of oligomeric aggregates that stayed in the cell.

It was recently shown by Petersen *et al.*[190] that besides binding to the LDL receptor family members, RAP also binds to calmodulin, an intracellular protein expressed at high levels in all cell types.

1.6.3. Ligand binding to LRP

A common property of the LDL Receptor family members is their ability to bind multiple ligands. Especially LRP binds a large number of structurally unrelated ligands (Table I) among which u-PA, t-PA, u-PA:PAI-1 and t-PA:PAI-1. The binding of all the ligands to LRP can be inhibited by RAP and is in most cases calcium dependent.[173] Moestrup *et al.*[191] showed that there are 8 binding sites for Ca^{2+} per molecule of LRP. The u-PA:PAI-1 complex is recognized by purified LRP with high affinity (K_d = 3 nM)[192], scu-PA and tcu-PA are recognized with 15-20 fold weaker affinity by LRP (K_d = 45, 60 nM respectively). Experiments showing that HepG2 cells rapidly internalized and degraded scu-PA independently of PAI-1 confirmed that LRP is able to mediate the cellular catabolism of scu-PA. The process could be inhibited by RAP and anti-LRP antibodies.[192] The second cluster of 8 complement-type repeats (CRD-II: residue 776-1399) mediates the binding of u-PA and u-PA:PAI-1.[159,193] Horn *et al.*[194] constructed fragments of the CRD-II domain of LRP, and showed that scu-PA is recognized by the sixth, seventh and eighth complement repeat in CRD-II, while RAP is recognized by the complement repeats 3, 4 and 5. The PAI-1 recognition site within CRD-II is present on EGF-4 and complement repeat four, five, six and seven. Since scu-PA and RAP are recognized by different complement repeats within CRD-II the inhibition of the binding of scu-PA to LRP by RAP probably is caused by steric hindrance.

A multipoint attachment model for the binding of tcu-PA and tcu-PA:PAI-1 to LRP has been described by Nykjaer *et al.*[195] Binding of tcu-PA to purified LRP occurs via low affinity recognition sites in the ATF-domain (K_i = 1050 nM) and the protease domain of u-PA (K_i = 850 nM). PAI-1 binds to LRP with a K_i of 55 nM. When complexed, the affinity for LRP increases (K_i = 0.4 nM) with a "bonus effect" on affinity because multiple sites are available in the same complex.

Besides recognition by LRP, u-PA is also recognized by gp330 and the VLDLr. Purified gp330 binds to scu-PA, PAI-1, tcu-PA and tcu-PA:PAI-1 with high affinity (K_d = 18.4, 11.0, 10.3 and 1.1 nM respectively) and mediates their internalization and degradation in cultured type II pneumocytes.[196] The VLDLr also binds tcu-PA:PAI-1 with high affinity[197], the binding of DFP-treated tcu-PA, PAI-1, tcu-PA:PAI-2 is weak or absent.

Table I: Ligands for LRP.

α_2-M-proteinase complexes[198,199]	apoE[210]
PZP-proteinase complexes[200]	apoE-enriched ß-VLDL[211]
t-PA[201]	lipoprotein lipase (LPL)[212,213]
u-PA[192]	LPL-enriched VLDL[214]
t-PA:PAI-1[202]	LPL-enriched ß-VLDL[215]
u-PA:PAI-1[203,204]	lactoferrin[215]
u-PA:protease nexin I[205]	hepatic lipase[216]
tissue factor pathway inhibitor[206]	pseudomonas exotoxin A[217]
thrombospondin[207,208]	minor group of human rhinovirus[218]
plant ribosome-inactivating protein[209]	malaria circumsporozoite protein[219]
RAP[176]	

1.6.4. LRP mediated internalization of u-PA:PAI-1 in the presence of uPAR

A model for the events at the surface of cells expressing equal amounts of both uPAR and LRP is postulated by Nykjaer *et al.*[195] HMW-scu-PA may bind both to uPAR and LRP, but due to a higher affinity for uPAR than for LRP binding to uPAR is preferred. UPAR bound HMW-scu-PA is not internalized but remains at the cell surface. Binding of HMW-scu-PA to LRP may function as a backup system for the clearance of HMW-scu-PA when uPAR is saturated. When bound to uPAR, HMW-scu-PA does not interact with LRP due to shielding of the ATF-domain of HMW-scu-PA. uPAR-bound HMW-scu-PA is subsequently activated by plasmin to the active HMW-tcu-PA. HMW-tcu-PA is in turn inactivated by the formation of HMW-tcu-PA:PAI-1 complexes. Finally, HMW-tcu-PA:PAI-1 associated with uPAR binds to LRP and the complex is internalized and degraded. It has been shown by a microscopy study of Conese *et al.*[220] that uPAR is also internalized together with LRP and HMW-tcu-PA:PAI-1. In cells expressing more LRP than uPAR the role of uPAR in the binding of HMW-scu-PA will be less pronounced.

1.7. The asialoglycoprotein receptor

The rat asialoglycoprotein receptor (ASGPr) is a hexamer, consisting of 3 proteins of 41.5 kDa (Rat Hepatic Lectin-1 (RHL-1)), 49 kDa (RHL-2) and 54 kDa (RHL-3).[221] RHL-2 and RHL-3 differ by the presence of different carbohydrate side chains attached to a common peptide backbone.[222] The stoichiometry of the different polypeptides in the oligomer is not clearly defined[222,223], but when isolated the RHL-1 protein is the most abundant.[221] It is described that the RHL-1 and RHL-2/3 polypeptides are each associated into homooligodimers, resulting in a

functional receptor protein that consists of a heterooligomeric complex of RHL-1 and RHL-2/3 multimers.[222,223] The ASGPr is located exclusively on the parenchymal liver cells[224] and recognizes ligands that bear galactose-terminal carbohydrate chains.[225] The recognition is dependent on calcium and mono-, bi-, tri- and tetra antennary galactose-terminal residues bind with increasing affinities, with dissociation constants of 10^{-3}, 10^{-6}, $5*10^{-9}$ and 10^{-9} M, respectively.[226]

1.8. Proteoglycans

Since the kringle- and protease domain of u-PA bind to heparin[87,227] HMW-scu-PA may interact with cell-surface proteoglycans. Proteoglycans are produced by most eukaryotic cells and are versatile components of the pericellular and extracellular matrices. These very large polyanions bind water and cations. Proteoglycans are important in determining the viscoelastic properties of joints and of other structures that are subject to mechanical deformation.[228] Proteoglycans are proteins that have one or more glycosaminoglycan chains attached. They have no unifying feature and display a great diversity of protein forms. Many proteoglycans contain distinct protein and glycosaminoglycan chains that have specific functional properties. Glycosaminoglycans are made up of disaccharide repeating units containing a derivative of an amino sugar, either glucosamine or galactosamine. At least one of the sugars in the disaccharide has a negatively charged carboxylate or sulfate group. The glycosaminoglycan side chains are covalently bound to the core protein and may be chondroitin sulfate, dermatan sulfate, keratan sulfate, heparan sulfate or heparin. Many proteoglycans carry two types of glycosaminoglycan side chains.[229] The glycosaminoglycan chains dominate the physical properties of the protein core to which they are attached.

A large number of heparan sulfate proteoglycans are present on rat parenchymal liver cells and only a few on endothelial and Kupffer cells.[230] It was shown by Weiner *et al.*[231] that freshly isolated rat parenchymal liver cells express syndecan-1, 2 and 4, and during cultivation these cells also start to express betaglycan.[232] Kupffer cells express syndecan-1, 3 and 4. Rat liver endothelial cells expose perlecan (a heparan sulfate proteoglycan) and decorin (contains a single chondroitin sulfate or dermatan sulfate chain) to their cell surface.[232] A large number of ligands are known to bind to proteoglycans, among which apoE[234] and lipoprotein lipase.[235]

1.9. Outline of the thesis

Thrombolytic therapy with urokinase-type plasminogen activator is accompanied by a rapid clearance of u-PA from the circulation. The main organ for the uptake of u-PA appears to be the liver. Because of the rapid disappearance from the blood circulation, large amounts of u-PA administered over a long period of time are required to obtain optimal clot lysis. The aim of this study is to investigate which receptor(s) are responsible for the liver recognition of u-PA. With the obtained knowledge it may be possible to develop specific inhibitors of the interaction of u-PA with the liver, resulting in an increased amount of exogenous and/or endogenous u-PA in the circulation. As a consequence this may lead to an improved thrombolytic therapy and/or prevention of thrombosis.

The second chapter describes the differential recognition of two types of human high molecular weight single-chain urokinase-type plasminogen activator (HMW-scu-PA) by rat parenchymal liver cells. Native, glycosylated HMW-scu-PA is recognized by a 'lectin like recognition site' while *E.coli* produced recombinant scu-PA (rscu-PA) is recognized by the Low Density Lipoprotein Receptor-related Protein (LRP). In chapter three the contribution of LRP in the liver uptake and plasma clearance of rscu-PA *in vivo* is further investigated. The identification of the asialoglycoprotein receptor as the 'lectin like recognition site' for glycosylated high molecular weight two-chain u-PA (tcu-PA) derived from human urine is described in chapter four. This chapter also describes that besides by being recognized by the asialoglycoprotein receptor, urinary tcu-PA is recognized via its protein moiety by heparan sulfate proteoglycans. Since urinary urokinase displays a heterogeneity in its carbohydrates, we separated urinary tcu-PA in a fraction that contained GalNAcß1-4GlcNAcß1-2Man terminal bi- and triantennary glycans and a fraction that contained sulfated GalNAcß1-4GlcNAcß1-2Man terminal bi- and triantennary glycans. Differences in recognition of sulfated and non-sulfated GalNAcß1-4GlcNAcß1-2Man terminal tcu-PA were determined in an *in vitro* binding assay using the isolated asialoglycoprotein receptor (chapter five). The binding of sulfated and non-sulfated tcu-PA to the rat asialoglycoprotein receptor and heparan sulfate proteoglycans on parenchymal liver cells *in vitro* and *in vivo* is further established in chapter six. In chapter seven, the effect of complex formation of tcu-PA with its plasma inhibitor PAI-1 on the liver recognition is described. Additional information on the parenchymal liver cell recognition of LRP ligands is obtained with modified α_2-macroglobulin (chapter eight).

The results obtained in this study are reviewed and discussed with respect to the improvement of thrombolytic therapy in chapter nine.

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Chapter 2

Native and non-glycosylated recombinant single-chain urokinase-type plasminogen activator are recognized by different receptor systems on rat parenchymal liver cells

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SUMMARY

The recognition systems mediating the clearance of glycosylated high molecular weight single-chain urokinase-type plasminogen activator (HMW-scu-PA, produced in human embryonic kidney cells) and recombinant non-glycosylated scu-PA (rscu-PA, produced in *E.coli*) were analyzed by studying their binding characteristics to freshly isolated rat parenchymal liver cells.

The binding of ^{125}I-HMW-scu-PA at 4 °C was calcium-dependent and of high affinity (K_i= 37.6 nM) and could be inhibited by low molecular weight two-chain u-PA (LMW-tcu-PA) and lactose, but not by the Low Density Lipoprotein Receptor-related Protein (LRP)-associated 39-kDa protein (RAP), rscu-PA or a mutant form lacking amino acids 11-135 (delta125-rscu-PA). Removal of the carbohydrate side chain of HMW-scu-PA by treatment with N-glycosidase F, completely reduced the specific binding to the parenchymal cells and strongly reduced its competition with ^{125}I-HMW-scu-PA in cell binding.

Recombinant scu-PA also bound with high affinity (K_i = 38.7 nM) to the parenchymal liver cells. The binding of ^{125}I-rscu-PA could be competed for by unlabelled rscu-PA while delta125-rscu-PA, LMW-tcu-PA or lactose were ineffective. In contrast to HMW-scu-PA, binding of ^{125}I-rscu-PA could be effectively inhibited by RAP (K_i = 1.1 nM), while also its association and degradation, as determined at 37 °C, were inhibited by RAP. Pretreatment of the parenchymal cells with proteinase K supplied further evidence for the involvement of two different receptor systems. The binding of rscu-PA was decreased for 91 %, while that of HMW-scu-PA showed a decrease of 51 %.

It is suggested that native HMW-scu-PA is bound and degraded by the rat parenchymal liver cells via a lectin-like recognition site, while non-glycosylated recombinant scu-PA is bound and degraded by rat parenchymal liver cells via the Low Density Lipoprotein Receptor-related Protein (LRP). The differences in recognition system for native and recombinant proteins by liver cells suggest that the glycosylation of recombinant proteins, as obtained in mammalian expression systems, can be important for their physiological fate and their pharmacological application.

INTRODUCTION

Urokinase-type plasminogen activator (u-PA) is produced as a single chain protein (scu-PA) with a molecular weight of 55,000 by e.g. kidney cells[1,2], many tumor cells[3] and fibroblasts.[4] Proteolytic cleavage of scu-PA between Lys_{158} and Ile_{159} by plasmin or kallikrein results in the conversion into an active two-chain form (tcu-PA). A low molecular weight form of scu-PA (LMW-scu-PA), lacking the first 143 N-terminal amino acids, has also been isolated.[2,5]

Because of its higher fibrin-selectivity[2,6,7], affinity for plasminogen[8-10] and better stability in plasma[11] scu-PA is a more suitable candidate for thrombolytic therapy then tcu-PA. One drawback of the use of scu-PA as thrombolytic agent is its rapid clearance from the circulation.[5,12,13] The liver and to a lesser extent the kidney have been identified to play a major role in the uptake of HMW- and LMW-scu-PA in monkeys, rabbits and rats.[5,12-15] We have described on rat parenchymal liver cells a common high affinity binding site for HMW-scu-PA and LMW-scu-PA.[15] This binding site is different from the u-PA-receptor that has been described on human monocytes and tumor cells.[16-18] This human receptor is a heavily glycosylated protein with a *Mr* of 55,000-60,000[18,19], which is attached to the cell membrane via a glycosyl-phosphadityl inositol (GPI) anchor.[20] It recognizes the epidermal growth factor domain (residue 5-45) in u-PA and therefore interacts only with HMW-scu-PA.[21] Binding of HMW-scu-PA to this receptor is not coupled to internalization and degradation[16,17], while with the receptor on rat parenchymal liver cells, binding is coupled to internalization and degradation of HMW- and LMW-scu-PA.[15]

Recently it has been shown by several groups that u-PA and u-PA:PAI-1 complexes can bind to the Low Density Lipoprotein Receptor-related Protein/α_2-Macroglobulin Receptor (LRP/α_2-MR) after which internalization and degradation occurs.[22-24] This has led to the concept that scu-PA or tcu-PA:PAI initially binds to the u-PA receptor, is then transferred to LRP/α_2-MR, and is finally internalized and degraded. The relevance of this system for parenchymal liver cells, which *in vivo* are mainly responsible for the catabolism of scu-PA, is however unclear.

LRP is a 600-kDa cell surface receptor that is present in a broad spectrum of cell types, e.g. hepatocytes, macrophages and fibroblasts.[25-28] A 39-kDa Receptor-Associated Protein (RAP) is non-covalently associated with LRP. LRP binds several apparently unrelated ligands: RAP, α_2-M-proteinase complexes[25], apoE enriched ß-migrating Very-Low Density Lipoprotein (ß-VLDL)[29], lipoprotein lipase[30], tissue-type plasminogen activator (t-PA)[31], t-PA:PAI-1 and u-PA:PAI-1 complexes.[22,32,33] Binding of all these ligands can be blocked by RAP.

In the present study, we characterized on freshly isolated rat parenchymal liver cells the recognition systems for two types of scu-PA, native HMW-scu-PA and recombinant non-glycosylated scu-PA (rscu-PA). The results suggest that rscu-PA is bound and degraded via LRP, whereas glycosylated native HMW-scu-PA is bound and degraded via a lectin-like recognition system.

MATERIALS AND METHODS

Materials

HMW-scu-PA and LMW-tcu-PA ("Abbokinase") from human embryonic kidney cell cultures were generous gifts from Dr. J. Henkin, Abbott Laboratories (Abbott Park, Il.).[34] Rscu-PA and

delta125-rscu-PA, produced in *E.coli*, were generous gifts from Dr. A. Molinari, Farmitalia Carlo Erba (Milan, Italy).[35] HMW-tcu-PA was commercially available "Ukidan" from Serono (Aubonne, Switzerland). GST-RAP, a conjugate of RAP and glutathione S-transferase[36] produced in *E.coli*[37] showing similar binding characteristics as RAP, was a generous gift from Dr. D.K. Strickland. Proteinase K and N-glycosidase F were from Boehringer Mannheim, 2-(4-(-Hydroxyethyl)-1-piperazinyl)-ethanesulfonic acid (HEPES) was obtained from Merck (Darmstadt, Germany). Bovine serum albumin (BSA fraction V) and collagenase type IV were from Sigma (St. Louis, MO, U.S.A.). Ethylene glycol-O,O'-bis(2-aminoethyl)-N,N,N',N'-tetraacetic acid (EGTA) was from Fluka (Buchs, Switzerland). Dulbecco's modified Eagle medium (DMEM) was from Gibco (Irvine, Scotland). ^{125}I (carrier free) in NaOH was obtained from Amersham, (Buckinghamshire, United Kingdom). All other chemicals were of analytical grade.

Radiolabeling of urokinase

HMW-scu-PA and rscu-PA were labelled with ^{125}I using the iodogen method[38], resulting in a specific radioactivity of 5000 cpm/ng (HMW-scu-PA) and 2500 cpm/ng (rscu-PA), without loss of latent enzyme activity or aggregation.[15]

Isolation of parenchymal liver cells

Male Wistar rats (250-300 g) were anaesthetized and parenchymal liver cells were isolated by perfusion of the liver for 9 min with collagenase (type IV, 0.05% (w/v)) at 37 °C by the method of Seglen[39], modified as described previously.[40] The obtained parenchymal liver cells were ≥ 95% viable as judged by 0.2 % trypan blue exclusion and ≥ 99 % pure as judged by light microscopy. For binding studies the parenchymal liver cells were resuspended in DMEM supplemented with 2 % BSA (pH 7.4).

In vitro binding studies

Parenchymal cells (1-2 mg cell protein/ml) were incubated in a volume of 0.5 ml with 1 nM ^{125}I-HMW-scu-PA or ^{125}I-rscu-PA with or without competitors at the indicated concentrations in DMEM containing 2 % (w/v) BSA pH 7.4. Incubations were carried out in plastic containers (Kartell, Milan, Italy) for 2 h at 4 °C under continuous shaking (150 rpm, Adolf Kuhner, Basel, Switzerland). At the end of the incubations cells were washed twice at 4 °C for 30 sec at 50 x g with buffer containing 10 mM Tris-HCl, 0.15 M NaCl, 5 mM $CaCl_2$, 0.2 % BSA, pH 7.4 (at 4 °C), followed by one wash with the same buffer without BSA. Finally, cells were resuspended in this buffer (without BSA) and cell bound radioactivity was counted in a gamma counter. The amount of cell protein was determined as described by Lowry *et al.*[41] with BSA as standard.

Binding parameters (K_i) were determined as described before[15] from displacement curves using a sigmoid model of the binding data with a 'Hill slope" that varied from 0.85-1.0 representing a one-site binding model (Graphpad: H. Motulsky, ISI software, San Diego, U.S.A.).

Association and degradation *in vitro*

The association and degradation of 1 nM ^{125}I-HMW-scu-PA or ^{125}I-rscu-PA was determined at 37 °C at the indicated incubation times in a volume of 0.5 ml. Cells and medium were separated by centrifugation (30 sec 50 x g) and the cell pellets were washed twice at 4 °C with cold 10 mM Tris-HCl, 0.15 M NaCl, 5 mM $CaCl_2$, 0.2 % BSA, pH 7.4 (at 4 °C), followed by one wash with the same buffer without BSA. The cells were resuspended in the latter buffer and radioactivity was counted followed by determination of the amount of cell protein as described by Lowry *et al.*[41] with BSA as standard. Supernatants (400 μl) were precipitated by addition of 160 μl trichloroacetic acid 35 %. The degradation values represent total trichloroacetic acid soluble radioactivity.

Treatment of HMW-tcu-PA and HMW-scu-PA with N-glycosidase F

HMW-tcu-PA (Ukidan, 100 μg/ml) was treated for 18 h at 37 °C with 4 U/ml N-glycosidase F in 50 mM phosphate buffer, 10 mM EDTA, 10 mM NaN_3, pH 7.2, followed by dialysis against HANKS/0.16% Hepes/0.3% BSA pH 7.4. Removal of the carbohydrate moiety was checked by 10

% polyacrylamide gel electrophoresis containing sodium dodecyl sulphate (SDS-PAGE). Competition studies were carried out as described above, with 1 nM ^{125}I-HMW-scu-PA as ligand and N-glycosidase F treated HMW-tcu-PA as competitor. ^{125}I-HMW-scu-PA was deglycosylated similarly.

Treatment of cells with proteinase K

Isolated parenchymal liver cells (0.5 ml, approx. 2-3.10^6 cells, > 95 % viable) were incubated at 0 °C in DMEM pH 7.4 containing 2 mg/ml proteinase K. Every 2 min, the cells were shaken. At the indicated time, 100 μl of 20 % BSA was added and the cells were washed three times with DMEM containing 2 % BSA (pH 7.4). Analysis of the viability showed that 2 h after incubation of the cells with proteinase K > 90 % of the cells were viable. Finally, the cells were incubated for 2 h (at 4 °C) with 1 nM ^{125}I-rscu-PA or ^{125}I-HMW-scu-PA in DMEM containing 2 % BSA pH 7.4 under continuous shaking.

RESULTS

In vitro interaction of HMW-scu-PA and rscu-PA with liver cells: Binding and competition studies

Displacement studies of ^{125}I-HMW-scu-PA with unlabelled HMW-scu-PA showed an apparent K_i for the binding of HMW-scu-PA to rat parenchymal liver cells of 37.6 nM, with a low percentage of non-specific binding of 10 % (figure 1). The specific binding could be completely competed for by LMW-tcu-PA (K_i = 49.6 nM), indicating the involvement of a common receptor for both HMW- and LMW-u-PA, which is in agreement with previously published data.[15] The binding of ^{125}I-HMW-scu-PA could not be competed for by rscu-PA or delta125-rscu-PA (lacking residue 11-135) upto an excess of 455 nM.

The displacement of ^{125}I-rscu-PA by unlabelled rscu-PA indicated that rscu-PA binds with high affinity to the parenchymal liver cells (K_i = 38.7 nM) with a non-specific binding of 35 % (figure 2). The binding of ^{125}I-rscu-PA could not be competed for by delta125-rscu-PA. This indicates that probably a part of the A-chain of rscu-PA is recognized by the parenchymal cells. The binding site of ^{125}I-rscu-PA on parenchymal cells was not competed for by LMW-tcu-PA, but binding of ^{125}I-rscu-PA could be competed for, with a relatively low effectivity, by HMW-scu-PA (K_i = 176 nM). This indicates that HMW-scu-PA is able to compete for the same recognition site on the parenchymal cells as ^{125}I-rscu-PA but with an approx. 12 times lower affinity.

For reason that HMW-scu-PA and rscu-PA do differ in the presence and absence of carbohydrate moieties respectively, a competition study with D-lactose was performed. Figure 3 shows that the binding of ^{125}I-rscu-PA is not sensitive for lactose competition, while binding of HMW-scu-PA to parenchymal cells could be displaced for 95 % by D-lactose (K_i = 8.2 mM).

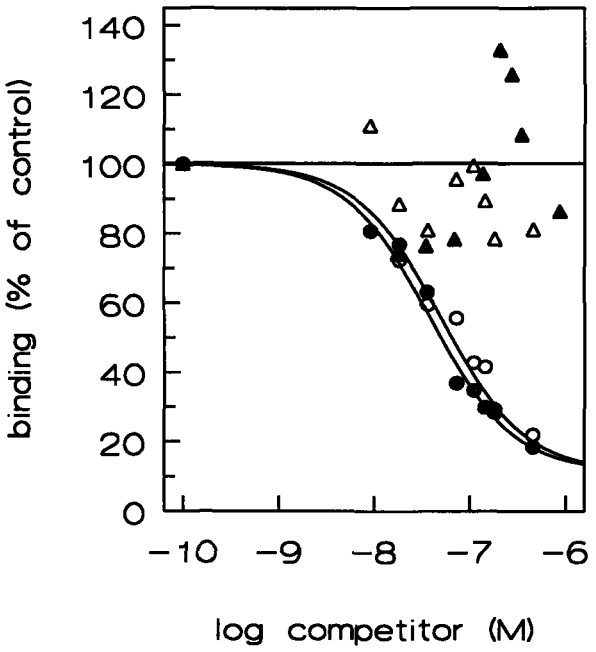

Figure 1: Effect of unlabelled HMW-scu-PA, LMW-tcu-PA, rscu-PA or delta125-rscu-PA on the binding of ^{125}I-HMW-scu-PA by rat parenchymal liver cells *in vitro*. Freshly isolated rat parenchymal liver cells were incubated for 2 h at 4 °C in the presence of ^{125}I-HMW-scu-PA with increasing amounts of unlabelled HMW-scu-PA (●) or LMW-tcu-PA (O), rscu-PA (▲) or delta125-rscu-PA (Δ). Data are expressed as % of control binding (43.1 ± 6.2 fmol/mg cell protein). Data represent the mean of 2 experiments and for all the data points errors present less than 10 %.

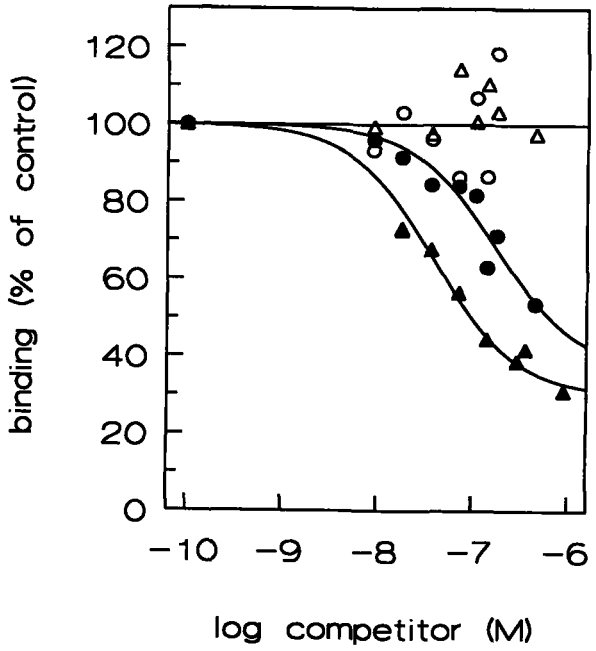

Figure 2: Effect of unlabelled HMW-scu-PA, LMW-tcu-PA, rscu-PA or delta125-rscu-PA on the binding of ^{125}I-rscu-PA by rat parenchymal liver cells *in vitro*. Freshly isolated rat parenchymal liver cells were incubated for 2 h at 4 °C in the presence of ^{125}I-rscu-PA with increasing amounts of rscu-PA (▲), delta125-rscu-PA (Δ), HMW-scu-PA (●) or LMW-tcu-PA (O). Data are expressed as % of control binding (24.7 ± 1.1 fmol/mg cell protein). Data represent the mean of 2 experiments and for all the data points errors present less than 10 %.

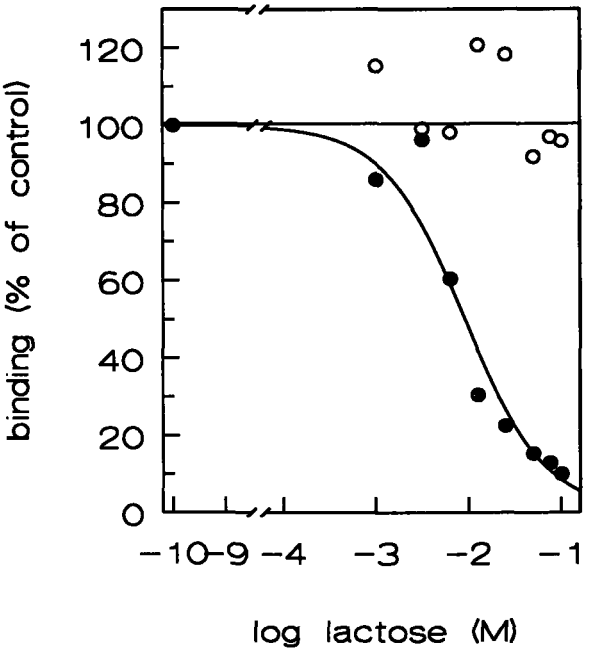

Figure 3: Effect of D-lactose on the binding of ^{125}I-HMW-scu-PA and ^{125}I-rscu-PA by parenchymal liver cells. Rat parenchymal cells were incubated for 2 h at 4 °C with 1 nM ^{125}I-HMW-scu-PA (●) or ^{125}I-rscu-PA (O) and with the indicated concentrations of D-lactose. Data represent the mean of 2 experiments and for all the data points errors present less than 10 %. The 100 % binding values were similar as described in figure 1 and 2.

Calcium dependency of the binding

Various recognition systems differ in their dependency on Ca^{2+}. Therefore the binding of HMW-scu-PA and rscu-PA to freshly isolated parenchymal liver cells was determined in the presence or absence of Ca^{2+} (figure 4). Binding of HMW-scu-PA to parenchymal cells was strictly Ca^{2+}-dependent and in the absence of Ca^{2+} the binding is only 15 % of the maximum level in the presence of Ca^{2+}. The binding of rscu-PA in the absence of Ca^{2+}, however, is still more than 60 % of the level in the presence of Ca^{2+}.

Competition of rscu-PA and HMW-scu-PA by GST-RAP

The potential involvement of LRP in the binding of HMW-scu-PA or rscu-PA to parenchymal liver cells was studied by investigating the effect of increasing concentrations of the Receptor-Associated Protein (RAP) on the binding of both HMW-scu-PA and rscu-PA (figure 5). The binding of native HMW-scu-PA to parenchymal cells was not affected by the addition of GST-RAP in concentrations up to 78 nM. In contrast, the binding of rscu-PA to parenchymal cells was for 80 % sensitive to GST-RAP, with a K_i = 1.1 nM. The GST moiety did not contribute to

the capacity of GST-RAP to inhibit the binding of rscu-PA to LRP because experiments with RAP gave the same results (data not shown).

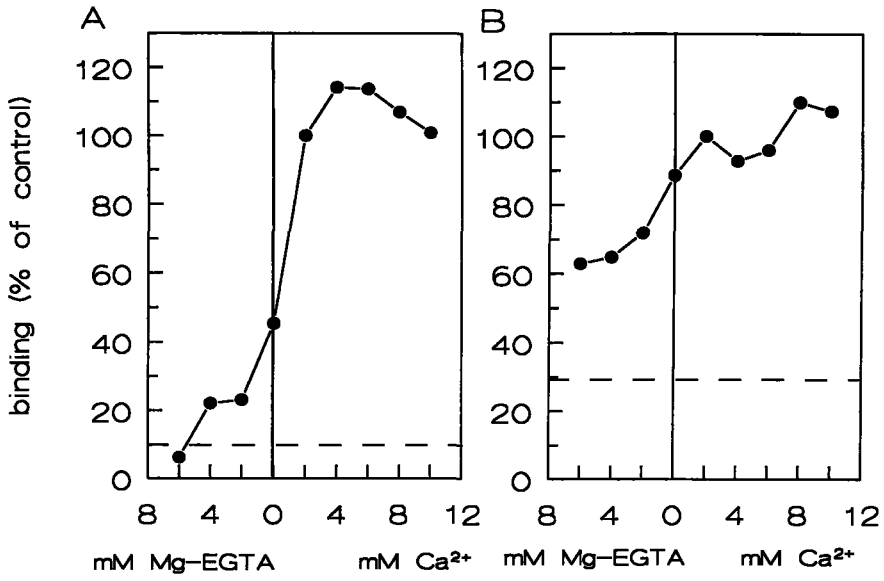

Figure 4: The effect of varying Ca^{2+} or EGTA concentrations on the binding of ^{125}I-HMW-scu-PA and ^{125}I-rscu-PA to parenchymal liver cells. Rat parenchymal cells were incubated for 2 h at 4 °C with 1 nM ^{125}I-HMW-scu-PA (panel A) or ^{125}I-rscu-PA (panel B) and with the indicated concentrations of added Ca^{2+} or Mg-EGTA. The binding in DMEM (containing 2 mM Ca^{2+}) is taken as the 100 % value. The non-specific binding is represented in a dotted line and was the same in the presence of Ca^{2+} or EGTA. Data represent the mean of 2 experiments and for all the data points errors present less than 10 %. The 100 % binding values are described in figure 1 and 2.

Association and degradation: influence of GST-RAP

To further investigate the role of LRP in the association and degradation of HMW-scu-PA and rscu-PA, experiments were performed at 37 °C in the absence and presence of 140 nM GST-RAP. Figure 6 shows the time course of cell-association and -degradation of 1 nM ^{125}I-HMW-scu-PA (panel A) and ^{125}I-rscu-PA (panel B). The rate of cell-association of HMW-scu-PA is very rapid and at 10 min of incubation already 42.7 ± 2.7 fmol HMW-scu-PA/mg cell protein was associated

(= 93 % of the maximum amount of association). The degradation of HMW-scu-PA was relatively low: 9.6 ± 1.5 fmol/mg cell protein after 60 min of incubation, which is 22 % of the amount that became associated. Addition of 140 nM GST-RAP had no effect on the association and degradation of HMW-scu-PA. ^{125}I-rscu-PA also became rapidly associated with the parenchymal cells (25.9 ± 1.0 fmol/mg cell protein after 10 min of incubation, (= 98 % of the maximum amount)). Degradation reached a value of 8.0 ± 0.5 fmol/mg cell protein after 60 min of incubation (= 31 % of the maximum amount of cell association). In contrast to the results obtained with HMW-scu-PA, GST-RAP significantly inhibited the association of rscu-PA (to 5.5 ± 0.8 fmol rscu-PA/mg cell protein after 10 min of incubation), which is a reduction of approx. 79 %. Degradation of rscu-PA in the presence of GST-RAP was reduced by 41 % after 60 min of incubation. In the absence of cells no degradation was observed of ^{125}I-HMW-scu-PA and ^{125}I-rscu-PA (data not shown).

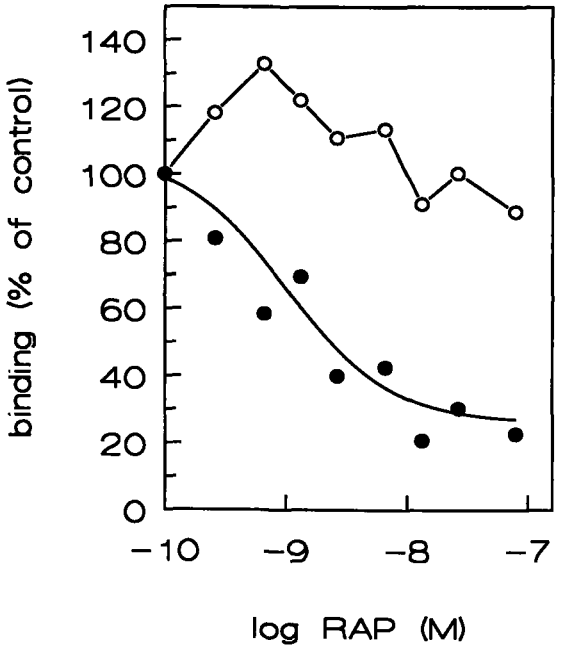

Figure 5: Effect of GST-RAP on the binding of rscu-PA and HMW-scu-PA to isolated parenchymal liver cells. Freshly isolated parenchymal cells were incubated for 2 h at 4 °C with 1 nM ^{125}I-HMW-scu-PA (○) or ^{125}I-rscu-PA (●) in the presence of the indicated concentrations of GST-RAP. Data are expressed as percentage of control binding, and represent the mean of 2 experiments. For all data points errors present less than 10 %. The 100 % binding values are described in figure 1 and 2.

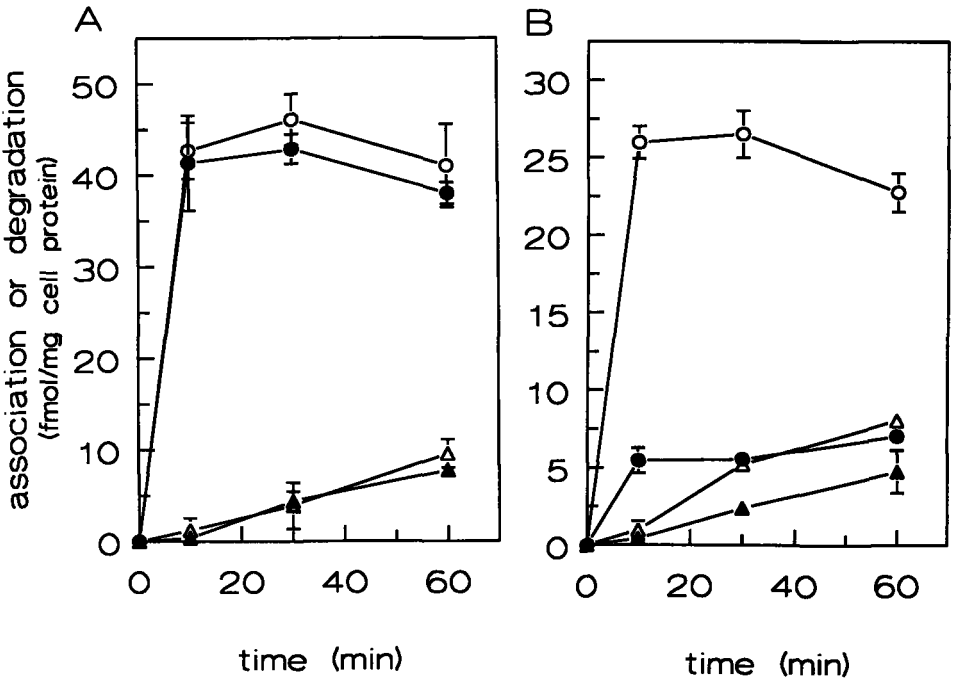

Figure 6: Association and degradation of HMW-scu-PA and rscu-PA by isolated parenchymal liver cells: effect of GST-RAP. Freshly isolated parenchymal cells were incubated with ^{125}I-HMW-scu-PA (panel A) or ^{125}I-rscu-PA (panel B) at a concentration of 1 nM at 37 °C. At the indicated times the association (○ = control, ● = + GST-RAP) and degradation (Δ = control, ▲ = + GST-RAP) were determined. Values are expressed as fmol of ligand associated or degraded/mg of cell protein. Values represent mean ± S.E.M. of 4 experiments. When error bars are not visible, errors are within symbol size.

Treatment with N-glycosidase F: role of the carbohydrate side chain in the binding of HMW-scu-PA

To investigate the possible participation of the carbohydrate side chain of u-PA in the binding to parenchymal cells, HMW-tcu-PA was treated with N-glycosidase F for 18 hours at 37 °C, subsequently its ability to compete with untreated ^{125}I-HMW-scu-PA was studied. Figure 7 shows that N-glycosidase F treated HMW-tcu-PA displaced ^{125}I-HMW-scu-PA for only 25 %, while glycosylated HMW-tcu-PA displaced ^{125}I-HMW-scu-PA for 75 %, implicating a marked reduction in its ability to compete with ^{125}I-HMW-scu-PA.

The removal of the carbohydrate side chain of HMW-scu-PA influenced its effectivity to bind to the parenchymal cells. Treatment of ^{125}I-HMW-scu-PA with N-

glycosidase F resulted in a 7.4 fold decrease in binding to the parenchymal cells (binding of control ^{125}I-HMW-scu-PA: 41.1 ± 3.2 fmol/mg cell protein, N-glycosidase F treated ^{125}I-HMW-scu-PA: 5.6 ± 1.6 fmol/mg cell protein). The level of binding of deglycosylated HMW-scu-PA was at a level comparable to the level of non-specific binding of HMW-scu-PA (6.5 ± 0.6 fmol/mg cell protein).

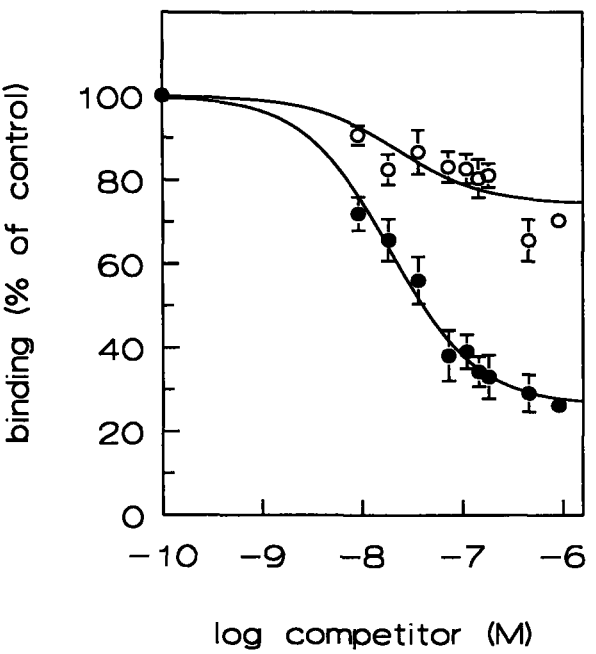

Figure 7: Role of the carbohydrate side chain of HMW-scu-PA in the binding to parenchymal liver cells. Parenchymal cells were incubated with 1 nM ^{125}I-HMW-scu-PA and the indicated concentrations of HMW-tcu-PA (●) or HMW-tcu-PA incubated for 18 h at 37 °C with 4 U/ml N-glycosidase F (○). Data are expressed as percentage of control binding, and represent the mean of 2 experiments. For all the data points errors present less than 10 %. The 100 % binding value is described in figure 1.

Effect of treatment of freshly isolated rat parenchymal cells with proteinase K on the binding of HMW-scu-PA and rscu-PA

As is described by van Dijk *et al.*[42], the binding of α_2-M-trypsin to LRP/α_2-MR is sensitive towards degradation with the enzyme proteinase K. Therefore we further investigated the nature of the binding sites for rscu-PA and HMW-scu-PA by preincubation of the cells for various periods of time with proteinase K, followed by analysis of the binding of rscu-PA or HMW-scu-PA. Figure 8 shows that the recognition site for HMW-scu-PA was more readily affected by proteinase K treatment of the cells than the recognition site for rscu-PA. However, incubation of parenchymal cells with proteinase K for 120 min led to a decrease of the binding of rscu-PA for 91 %, while that of HMW-scu-PA was decreased for 51 %, indicating that both recognition systems were not equally sensitive to proteinase K.

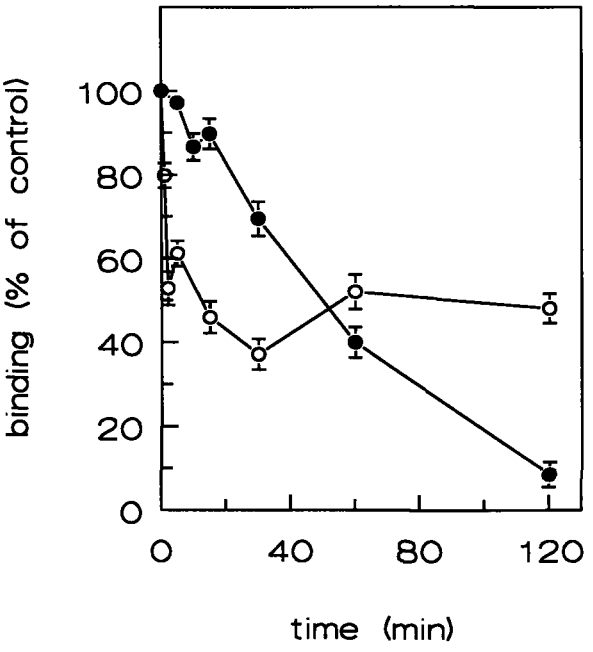

Figure 8: Effect of proteinase K treatment of parenchymal cells on the binding of HMW-scu-PA and rscu-PA. Freshly isolated rat parenchymal cells were incubated for the indicated period of time with 2 mg/ml proteinase K at 0 °C. After thoroughly washing, the cells were incubated for 2 h at 4 °C with 1 nM of ^{125}I-HMW-scu-PA (○) or ^{125}I-rscu-PA (●). The 100 % binding values are described in fig. 1 and 2. Data are expressed as percentage of control binding, and represent the mean of 2 experiments. For all the data points errors present less than 10 %.

DISCUSSION

Upon *in vivo* injection of native HMW-scu-PA and LMW-scu-PA into rats a rapid decay from the blood circulation has been observed for which the liver parenchymal cells are mainly responsible.[15] A specific receptor for native HMW-scu-PA and LMW-scu-PA has been found on isolated rat parenchymal liver cells[15], that is different from the well described u-PA-receptor which is present on human monocytes and tumor cells.[15-17] In order to further characterize the liver uptake system, we investigated the binding to parenchymal cells of two different types of single-chain urokinase type-plasminogen activator, native HMW-scu-PA produced in human embryonic kidney cells and recombinant non-glycosylated HMW-scu-PA produced in *E.coli*. Unexpectedly we observed that the two types of u-PA bound with different properties to receptor sites on rat parenchymal liver cells, although both u-PA forms do contain the same HMW-scu-PA protein chain. The binding of native HMW-scu-PA to parenchymal liver cells was of high affinity (K_i = 37.6 nM), calcium-dependent and could be inhibited by LMW-tcu-PA (K_i = 49.6 nM), which corresponds with the previous findings[15] that the recognition site for native scu-PA is located in residue 144-411 of the molecule, which is in contrast to the human

monocytic u-PA receptor that binds to the growth factor domain of u-PA (residue 5-45). The fact that lactose affected the binding of HMW-scu-PA to parenchymal liver cells indicates that the binding site for native HMW-scu-PA may be a lectin-like molecule.[42] This could explain why recombinant non-glycosylated HMW-scu-PA does not interact with this binding site.

Further evidence for recognition of HMW-scu-PA by a lectin-like site is provided by the fact that removal of the carbohydrate side chain with N-glycosidase F reduced the binding of deglycosylated HMW-scu-PA to the parenchymal cells to 15 % of the control value. In addition, deglycosylated HMW-tcu-PA showed a strongly diminished reduction in the capacity to compete for native untreated ^{125}I-HMW-scu-PA.

Recombinant single-chain urokinase (rscu-PA) showed a comparable affinity for parenchymal liver cells as HMW-scu-PA (K_i = 38.7 nM), but the binding of rscu-PA was less influenced by Ca^{2+}. Parenchymal cell bound ^{125}I-rscu-PA could not be displaced by delta125-rscu-PA or lactose. This implies that the receptor recognition site of rscu-PA is located in the A-chain of the molecule, and that the receptor properties do not point to a lectin as primary interaction site.

As shown by others[22-24], u-PA and u-PA:PAI-1 complexes can bind to purified LRP. In HepG2 cells scu-PA is internalized and degraded after binding to LRP independently of PAI-1.[23] For monocytes it is suggested that LRP mediates the internalization and degradation of u-PA-receptor bound u-PA:PAI-1.[22] We observed in competition experiments with RAP and by investigating the effect of RAP on the association and degradation of rscu-PA that rscu-PA was recognized by LRP also in the absence of PAI-1. The binding of rscu-PA to LRP on rat parenchymal liver cells is less dependent on the presence of Ca^{2+} than is described for other ligands of LRP such as α_2-M-proteinase, t-PA:PAI-1, u-PA:PAI-1.[24,32,36,37] The dependency of Ca^{2+} for the binding of RAP to LRP is not clear. Moestrup and Gliemann[26] reported that EDTA did not inhibit the binding of RAP to LRP in ligand blotting experiments. Williams *et al.*[37] and Herz *et al.*[36], however, showed that EDTA abolished the binding of RAP to isolated LRP completely. Preliminary data from our lab show that the binding of t-PA:PAI complexes and RAP to LRP on rat parenchymal liver cells is also Ca^{2+}-independent.[44,45]

HMW-scu-PA on the contrary, is not recognized by LRP at low concentrations (1 nM). Competition experiments with ^{125}I-rscu-PA and HMW-scu-PA as competitor showed that only at high concentrations HMW-scu-PA might be recognized by LRP with a low affinity. It remains to be established why HMW-scu-PA has a lower affinity for LRP than rscu-PA.

Further evidence for the involvement of two different receptors for rscu-PA and native HMW-scu-PA came from experiments in which parenchymal cells were pretreated with proteinase K. LRP is extremely sensitive to proteinase K as is described by van Dijk *et al.*[42], while the lectin that recognizes HMW-scu-PA is less sensitive to proteinase K.

Recently Hajjar *et al.*[46] have described that O-linked α-fucose may mediate binding and degradation of tissue-type plasminogen activator (t-PA) by HepG2 cells. HMW-scu-PA contains an epidermal growth factor domain (EGF)-based threonine-linked α-fucose homologous to that of t-PA.[47,48] The novel hepatic lectin they postulate, is not playing a role in the recognition of HMW-scu-PA by rat parenchymal liver cells since recognition of HMW-scu-PA occurs via residue 144-411. The fucose is present on threonine-18 in the A-chain of the u-PA molecule, which appears not to be involved in the recognition of HMW-scu-PA by the parenchymal liver cells. The molecular nature of the lectin-like specific receptor on parenchymal cells which is responsible for the removal of native HMW-scu-PA from the plasma remains to be established. HMW-scu-PA may be sialylated to a varying extent[49] and the presence of galactose as the terminal sugar of the oligosaccharide chain, may result in the binding of scu-PA to the asialoglycoprotein receptor.

The finding that recombinant scu-PA is bound and degraded by rat parenchymal liver cells via the Low Density Lipoprotein Receptor-related Protein (LRP), whereas glycosylated native HMW-scu-PA may be bound and degraded via a lectin-like receptor site, suggests that glycosylation of recombinant proteins may be important for their physiological fate.

ACKNOWLEDGEMENT

This study was supported by the Netherlands Heart Foundation (grant 92.139).

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Chapter 3

The role of the Low-Density Lipoprotein Receptor-related Protein (LRP) in the plasma clearance and liver uptake of recombinant single-chain urokinase-type plasminogen activator in rats

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SUMMARY

Urokinase-type plasminogen activator (u-PA) is used as a thrombolytic agent in the treatment of acute myocardial infarction. *In vitro*, recombinant single-chain u-PA (rscu-PA) expressed in *E.coli* is recognized by the Low-Density Lipoprotein Receptor-related Protein (LRP) on rat parenchymal liver cells. In this study we investigated the role of LRP in the liver uptake and plasma clearance of rscu-PA in rats. A preinjection of the LRP inhibitor GST-RAP reduced the maximal liver uptake of ^{125}I-rscu-PA at 5 min after injection from 50 to 30 % of the injected dose and decreased the clearance of rscu-PA from 2.37 ml/min to 1.58 ml/min. Parenchymal, Kupffer and endothelial cells were responsible for 40, 50 and 10 % of the liver uptake, respectively. The reduction in liver uptake of rscu-PA by the preinjection GST-RAP was caused by a 91 % and 62 % reduction in the uptake by parenchymal and Kupffer cells, respectively. In order to investigate the part of rscu-PA that accounted for the interaction with LRP, experiments were performed with a mutant of rscu-PA lacking residues 11-135 (=delta125-rscu-PA). Deletion of residues 11-135 resulted in a 80 % reduction in liver uptake and a 2.4 times slower clearance (0.97 ml/min). The parenchymal, Kupffer and endothelial cells were responsible for respectively 60, 33 and 7 % of the liver uptake of ^{125}I-delta125-rscu-PA. Preinjection of GST-RAP completely reduced the liver uptake of delta125-rscu-PA and reduced its clearance to 0.79 ml/min. Treatment of isolated Kupffer cells with PI-PLC reduced the binding of rscu-PA by 40 %, suggesting the involvement of the urokinase-type Plasminogen Activator Receptor (uPAR) in the recognition of rscu-PA. Our results demonstrate that *in vivo* LRP is responsible for more than 90 % of the parenchymal liver cell mediated uptake of rscu-PA and for 60 % of the Kupffer cell interaction. It is also suggested that uPAR is involved in the Kupffer cell recognition of rscu-PA.

INTRODUCTION

Urokinase-type plasminogen activator (u-PA) is a highly specific serine protease that converts plasminogen to plasmin. Plasmin is a broad spectrum protease that degrades fibrin and several other proteins. Because of its capacity to degrade fibrin via plasminogen activation, u-PA is an attractive thrombolytic agent. Urokinase is produced as a single-chain proenzyme (scu-PA) with a molecular weight of 55,000 by e.g. kidney cells[1,2], tumor cells[3] and fibroblasts.[4] Proteolytic cleavage of scu-PA between Lys_{158} and Ile_{159} by plasmin or kallikrein results in the conversion into an active two-chain molecule (tcu-PA). Due to its higher fibrin-selectivity[5,6], its affinity for plasminogen[7-9] and a better stability in plasma[10] scu-PA is a more suitable candidate for thrombolytic therapy than tcu-PA.[11,12] When used in thrombolytic therapy u-PA is cleared rapidly from the

circulation.[13,14] The liver and to a lesser extent the kidneys have been identified to play a major role in the removal of u-PA from the plasma in monkeys, rabbits and rats.[13-18]

Recently we have described that *E.coli* produced recombinant scu-PA (rscu-PA) is bound and degraded by freshly isolated rat parenchymal liver cells via the Low-Density Lipoprotein Receptor-related Protein/α_2-Macroglobulin Receptor (LRP/α_2-MR).[19] LRP is synthesized in the endoplasmic reticulum as a 600 kDa precursor. After it reaches the Golgi complex, the protein is cleaved to generate two subunits of approximately 515 and 85 kDa, which are noncovalently associated.[20,21] The α-chain (515 kDa) contains the ligand binding domains, whereas the ß-chain (85 kDa) contains a single membrane spanning domain and two cytoplasmic NPXY sequences necessary for localization to coated pits.[21] Both subunits remain associated to each other during trafficking to the cell surface. LRP is expressed by a broad spectrum of cell types, e.g. hepatocytes, macrophages and fibroblasts.[22-25] A 39-kDa receptor associated protein (RAP) is non-covalently associated with LRP. RAP functions intracellularly as a molecular chaperon for LRP by interacting with LRP and maintaining LRP in an inactive ligand binding state.[26] As RAP dissociates from LRP in the Golgi apparatus, LRP becomes active as it travels to the cell surface. LRP binds several apparently unrelated ligands, e.g. RAP, α_2-M-proteinase complexes[22], apo E enriched ß-migrating Very Low Density Lipoprotein (ß-VLDL)[27], lipoprotein lipase[28], *Pseudomonas* exotoxin A[29], tissue-type plasminogen activator (t-PA)[30], t-PA:PAI-1 and u-PA:PAI-1 complexes.[19,20,31-33] Binding of all of these ligands to LRP can be blocked by RAP. Recently Willnow *et al.*[34] have shown that RAP-deficient mice show a significant reduction in LRP expression, resulting in an impaired clearance of LRP-specific ligands by the liver.

In the present study we characterized the interaction of rscu-PA with LRP *in vivo*. We demonstrate that LRP is responsible for the parenchymal liver cell mediated uptake of rscu-PA and for 60 % of the Kupffer cell interaction of rscu-PA. It is also suggested that besides LRP, the urokinase-type Plasminogen Activator Receptor (uPAR) is involved in 40 % of the high affinity binding of rscu-PA to the Kupffer cells.

MATERIALS AND METHODS

Materials

Bovine serum albumin (BSA fraction V) and collagenase type I and IV were from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's modified Eagle medium (DMEM) was from Gibco (Irvine, Scotland). ^{125}I (carrier free) in NaOH was obtained from Amersham (Buckinghamshire, United Kingdom). Pronase, trypsin (EC 3.4.21.4) from bovine pancreas, phosphatidylinositol phospholipase C (PI-PLC) and soybean trypsin inhibitor were bought from Boehringer Mannheim (Mannheim, Germany). Bio-Gel A-1.5m was obtained from Bio-Rad (USA). Rscu-PA and delta125-rscu-PA produced in *E.coli* were generous gifts from Dr. A. Molinari, Farmitalia Carlo Erba (Milan,

Italy).[35] The GST-RAP expression plasmid, containing rat 39-kDa cDNA was kindly provided by Dr. J. Herz (Dallas, USA). *E.coli* strain DH5α was from Fabagen (Utrecht, the Netherlands). α_2-M was a kind gift of Dr. W. Boers, AMC (Amsterdam, the Netherlands).[36] All other chemicals were of analytical grade.

Protein iodination

Recombinant scu-PA was iodinated as described earlier[19] by the iodogen method, resulting in a specific radioactivity of approximately 2500 cpm/ng for rscu-PA and 1200 cpm/ng for delta125-rscu-PA. Human α_2-macroglobulin (α_2-M) was radioiodinated with chloramine T as published previously by Davidsen *et al.*[37] and subsequently activated with trypsin (^{125}I-α_2M-T)).

Production of GST-RAP

A plasmid (pGEX) encoding for a fusion protein (GST-RAP) of glutathione S-transferase (GST) and the 39 kDa protein or Receptor Associated Protein (RAP) was transformed in *E.coli* (DH5α). GST-RAP was produced as described by Herz *et al.*[38] The isolated GST-RAP was more than 95 % pure as determined by analysis on 10 % SDS-PAGE. The activity of GST-RAP was tested by determining the ability of GST-RAP to inhibit the binding of ^{125}I-α_2-M-T to freshly isolated rat parenchymal liver cells. The inhibitory constant for the displacement of the binding of ^{125}I-α_2-M-T by GST-RAP was 1 nM, which is similar to the K_d observed for GST-RAP kindly donated by Dr. D.K. Strickland (Rockville, Maryland, USA). The purified GST-RAP was dialysed against PBS before administration to rats.

Serum decay and tissue uptake

9-10 weeks old male Wistar rats (200-230 g, Broekman Institute BV, Someren, The Netherlands) were anaesthetized by intraperitoneal injection of 15-20 mg of sodium pentobarbital. The abdomen was opened, radiolabelled rscu-PA (1.5 μg/kg body weight (b.w.)), delta125-rscu-PA (2.5 μg/kg b.w.) or α_2-M-T (8 μg/kg b.w.) was injected via the inferior vena cava and at the indicated times liver lobules were tied off and 0.3 ml blood samples were taken from the inferior vena cava and allowed to clot for 30 min. The body temperature of the rats was kept at 36.5-37 °C using an infrared lamp. The blood samples were centrifuged for 2 min at 10,000 xg and 100 μl serum samples were counted for radioactivity. The excised liver lobules (which totally weighed less than 15 % of the liver mass) were weighted and the radioactivity was counted. Total liver uptake was determined by weighing the liver at the end of the experiment. The total amount of radioactivity in the serum and liver lobules was determined as described previously.[17,39,40] Uptake of ^{125}I-rscu-PA and ^{125}I-delta125-rscu-PA in other organs was determined by removal and weighing the organs at 7 min after injection. Radioactivity was counted and corrected for the radioactivity in the serum. Values for the serum content of tissues were calculated by determining the amounts of radioactivity present in tissues after injection of radiolabelled serum albumin.

Liver cell distribution of u-PA

Rats were anaesthetized and injected with radiolabelled ligands as described above. After 10 min circulation the liver was perfused at 8 °C with collagenase. Parenchymal (PC), endothelial (EC) and Kupffer cells (KC) were isolated by centrifugation, followed by counterflow centrifugal elutriation at 4 °C as described in detail elsewhere.[41-44] The contributions of the different liver cell types to the total hepatic uptake of the injected ligand were calculated with the assumption that parenchymal, endothelial and Kupffer cells account for 92.5, 3.3 and 2.5% of the total liver protein mass, respectively.[41-44] As found for a number of substrates[41-44], 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.

Isolation of Kupffer cells

Male Wistar rats were anaesthetized and Kupffer cells were isolated by perfusion of the liver

for 9 min with collagenase (type IV, 0.05 % (w/v)) at 37 °C by the method of Seglen[45], modified as described previously.[46] Liver cells were separated by differential centrifugation and the Kupffer cells were purified by counterflow elutriation as described in detail elsewhere.[44] The purity of the Kupffer cells as monitored by peroxidase staining was at least 95 % (0.1 % 3,3' diaminobenzidine in 0.05 M Tris-HCl, 7 % sucrose, 0.12 % (v/v) 30 % H_2O_2 pH 7.4; 20 min at 37 °C).

In vitro studies with freshly isolated Kupffer cells

Kupffer cells were incubated for 2 h at 37 °C with or without 3 U/ml PI-PLC in DMEM/2 %BSA pH 7.35. Incubations were carried out in plastic containers (Kartell, Milan Italy) under continuous shaking (150 rpm, Adolf Kuhner, Basel, Switzerland). At the end the cells were washed twice at 500 xg with DMEM/2%BSA to remove the PI-PLC. Cells ($1.5x10^6$; 150 μg of cell protein) were incubated for 2 h at 4 °C in a volume of 0.5 ml DMEM/2%BSA with 1 nM ^{125}I-rscu-PA or ^{125}I-delta125-rscu-PA. At the end of the incubation the cells were washed twice at 4 °C for 2 min at 500 xg with 10 mM Tris-HCl pH 7.35 supplemented with 0.15 M NaCl, 5 mM $CaCl_2$ and 0.2 % BSA, followed by one wash with the same buffer without BSA. Finally, cells were resuspended in this buffer and cell bound radioactivity was counted in a gamma counter. The amount of cell protein was determined as described by Lowry *et al.*[47] with BSA as standard.

For the study of the recognition of α_2-M-T by LRP, the Kupffer cells were incubated for 2 h at 4 °C with 4 nM ^{125}I-α_2-M-T and increasing concentrations of GST-RAP. At the end of the incubation the cells were washed as described above.

Pharmacokinetic analysis and statistics

Binding parameters (K_i) were determined from displacement curves using a sigmoid model of the binding data with a "Hill slope" of 0.9-1.0 representing a one site binding model (Graphpad Prism: 1994 Graphpad Software Inc., San Diego, U.S.A.). Plasma clearance curves were analyzed by computerized nonlinear fitting following a biphasic clearance model using the same program ($t_{1/2\alpha}$, $t_{1/2\beta}$, $AUC_{0\text{-}29}$). The clearance was calculated by the formula clearance = dose/$AUC_{0\text{-}29}$. Results are given as means ± S.D.

RESULTS

Serum decay, liver uptake and liver cell distribution of ^{125}I-rscu-PA

Upon injection of 1.5 μg ^{125}I-rscu-PA/kg body weight (b.w.) a rapid decay from the blood circulation was observed, and concurrently uptake in the liver (figure 1). A $t_{1/2\alpha}$ and $t_{1/2\beta}$ of 0.29 ± 0.01 min and 19.94 ± 4.90 min were found and a clearance of 2.37 ± 0.03 ml/min was determined (table I). Maximal liver uptake was reached at 5 min after injection (50.0 ± 1.6 %). Between 10 and 30 min after injection, the liver-associated radioactivity decreased to 17.7 ± 1.9 %, indicating degradation of rscu-PA.

In order to analyze the uptake of rscu-PA in liver parenchymal, endothelial and Kupffer cells, we isolated at 10 min after injection of ^{125}I-rscu-PA the different liver cell types. Figure 2 shows that the highest specific association of rscu-PA was observed with liver Kupffer cells (710.0 ± 60.6 % i.d. x 10^3/mg cell protein). A 44-fold lower amount of rscu-PA per mg cell protein was associated with the parenchymal cells (16.1 ± 3.6 % i.d. x 10^3/mg cell protein). The contribution of the various liver cell types to the total liver uptake of rscu-PA was calculated, taking

into account that parenchymal, Kupffer and endothelial cells contribute for respectively 92.5 %, 2.5 % and 3.3 % to the total amount of liver protein.[43,48,49] The parenchymal, Kupffer and endothelial cells contributed 40.4 %, 49.6 % and 10.0 % to the total liver uptake of ^{125}I-rscu-PA, respectively.

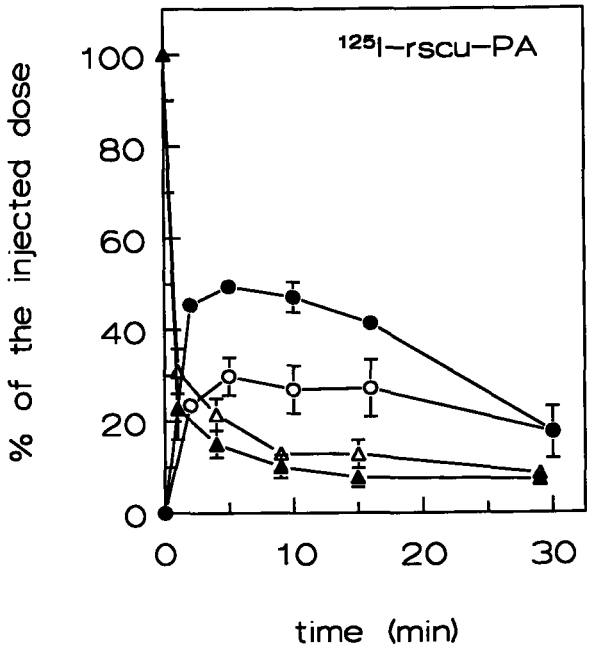

Figure 1. Effect of GST-RAP on the serum decay and liver uptake of ^{125}I-rscu-PA. Rats were injected intravenously with (open symbols) or without (closed symbols) 50 mg/kg body weight GST-RAP 1 min prior to injection of 1.5 μg/kg body weight ^{125}I-rscu-PA. At the indicated times after injection of the radiolabelled ligand, liver uptake (○,●) and serum decay (Δ,▲) were determined. Data expressed as % of the injected dose are mean ± S.D (n = 3).

Table I: Pharmacokinetic parameters from the clearance studies with ^{125}I-rscu-PA, ^{125}I-delta125-rscu-PA with and without a preinjection of GST-RAP.

ligand	t½α (min)	t½ß (min)	clearance (ml/min)
rscu-PA	0.29 ± 0.01 (84.4 ± 0.1%)	19.94 ± 4.90 (15.6 ± 0.1%)	2.37 ± 0.03
rscu-PA + GST-RAP	0.32 ± 0.02 (77.1 ± 4.2%)	17.83 ± 3.09 (22.8 ± 4.2%)	1.58 ± 0.27
delta125-rscu-PA	1.34 ± 0.01 (66.4 ± 4.2%)	37.72 ± 6.03 (32.2 ± 4.2%)	0.97 ± 0.04
delta125-rscu-PA + GST-RAP	0.60 ± 0.10 (46.7 ± 0.7%)	20.80 ± 1.52 (53.1 ± 0.6%)	0.79 ± 0.03

Pharmacokinetic parameters were derived from computer fitting as described in materials and methods. The clearance curves are shown in figure 1 and 5. () indicate the % of the injected dose that is cleared in the α or ß phase.

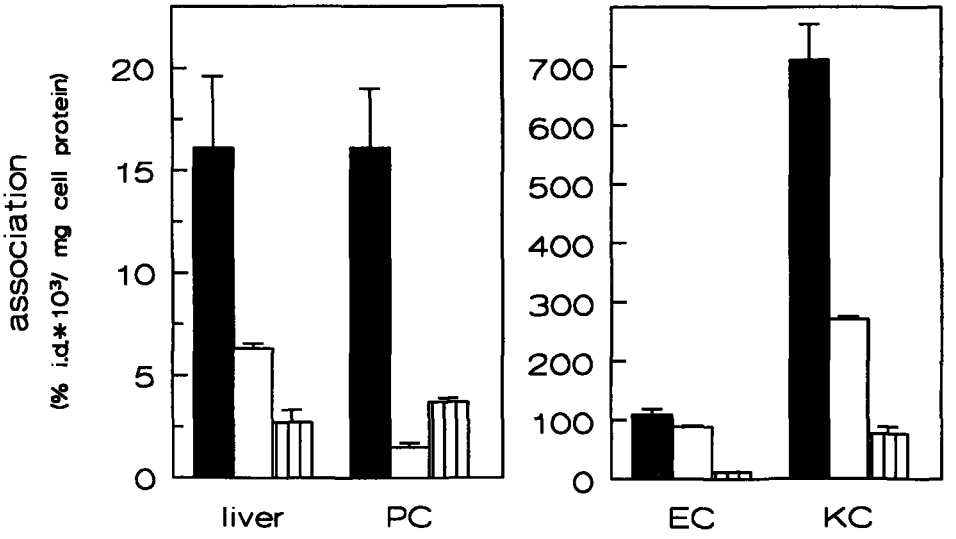

Figure 2. Contribution of the liver parenchymal, endothelial and Kupffer cells to the association of rscu-PA (control), rscu-PA with a preinjection of GST-RAP or delta125-rscu-PA. Rats were injected with 1.5 μg/kg body weight (b.w.) ^{125}I-rscu-PA (closed bars), with 50 mg/kg b.w. GST-RAP 1 min prior to ^{125}I-rscu-PA (open bars) or with 3 μg/kg b.w. ^{125}I-delta125-rscu-PA (striped bars). At 10 min after injection of the radiolabelled proteins the liver was perfused at 8 °C. After 8 min of perfusion, the total liver association was determined (liver) and subsequently parenchymal (PC), endothelial (EC) and Kupffer cells (KC) were isolated by a low temperature method. Data represent the mean of 2 experiments ± the variation of the individual experiments.

Effect of GST-RAP on the serum decay and liver uptake of α_2-Macroglobulin-T (α_2-M-T)

The effect of GST-RAP, an inhibitor of the interaction of various ligands with LRP/α_2-MR on the liver uptake and serum decay of ^{125}I-α_2-Macroglobulin-T (α_2-M-T) was determined. Figure 3 shows that ^{125}I-α_2-M-T was cleared rapidly from the circulation with a serum half-life of 1.31 ± 0.11 min for the α-phase and 22.35 ± 4.36 min for the ß-phase. A maximal liver uptake was observed at 10 min after injection ((80.8 ± 1.0 % of the injected dose). Preinjection of an increasing dose of GST-RAP reduced the maximal liver uptake of ^{125}I-α_2-M-T at 10 min after injection from 80.8 ± 1.0 % to 65.0 % (5 mg GST-RAP/kg body weight (b.w.)), 58.1 % (15 mg GST-RAP/kg b.w.) or 2.94 ± 0.04 % of the injected dose (50 mg GST-RAP/kg

b.w. (figure 3A)). Concurrently, the clearance of ^{125}I-α_2-M-T is slowed a 5.1-fold from 1.58 ± 0.01 ml/min (control) to 0.31 ± 0.01 ml/min (50 mg GST-RAP/kg b.w.). Since 50 mg of GST-RAP/kg b.w. resulted in a complete blockade of the interaction of ^{125}I-α_2-M-T with LRP, this dose was used in the rest of the experiments.

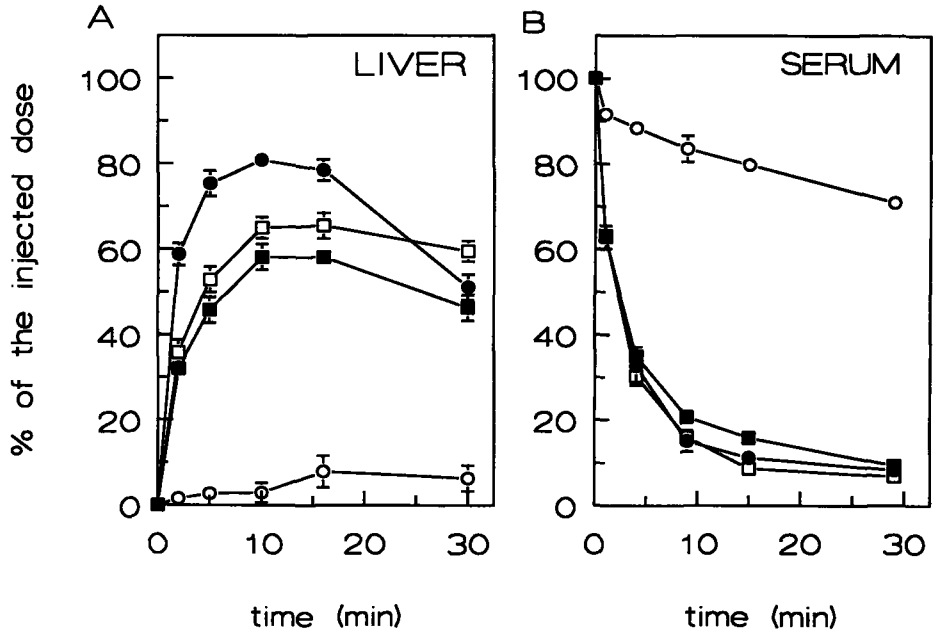

Figure 3. Effect of GST-RAP on the serum decay and liver uptake of α_2-Macroglobulin-T (α_2-M-T). Rats were injected intravenously with 0 (●), 5 (□), 15 (■) or 50 (○) mg/kg body weight GST-RAP 1 min prior to injection of 8 μg/kg body weight ^{125}I-α_2-M-T. At the indicated times after injection of the radiolabelled ligand, the liver uptake (A) and serum decay (B) were determined. Data expressed as % of the injected dose are mean ± S.D. (n = 3).

Effect of GST-RAP on the serum decay, liver uptake and liver cell distribution of ^{125}I-rscu-PA

In order to investigate the involvement of LRP in the liver uptake of rscu-PA, GST-RAP (50 mg/kg b.w.) was injected 1 min prior to ^{125}I-rscu-PA (figure 1). The maximal liver uptake at 5 min after injection was lowered from 50.0 ± 1.6 to 29.9 ± 4.1 % of the injected dose. The clearance was decreased 1.5-fold from 2.37 ±

0.03 ml/min (control) to 1.58 ± 0.27 ml/min (+GST-RAP, (table I)).

The effect of preinjection of GST-RAP on the cellular liver distribution of ^{125}I-rscu-PA was also studied (figure 2). The liver uptake of rscu-PA was reduced from 16.1 ± 4.5 % i.d/mg cell protein to 6.31 ± 0.24 % i.d/mg cell protein. The association of rscu-PA with the liver parenchymal cells was reduced 11-fold and the relative contribution of the parenchymal cells to the total liver uptake was reduced from 40.4 to 12.2 %. The Kupffer cell association of ^{125}I-rscu-PA was reduced 2.6-fold. The uptake of ^{125}I-rscu-PA by the endothelial cells was only slightly affected.

Effect of GST-RAP on the organ distribution of ^{125}I-rscu-PA

We determined whether other organs than the liver contributed to the removal of rscu-PA from the circulation (figure 4). Besides the liver (38.5 ± 0.2 % i.d.) the kidneys (3.0 ± 0.5 %), lungs (2.7 ± 1.1 %), muscles (4.1 ± 0.6 %) and skin (4.6 ± 2.0 %) contributed significantly to the serum decay at 7 min after injection of ^{125}I-rscu-PA.

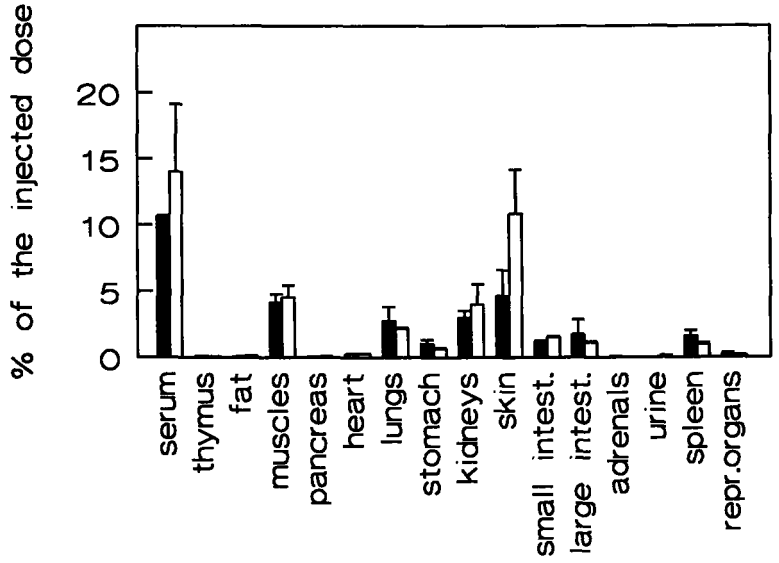

Figure 4. Tissue distribution of intravenously injected ^{125}I-rscu-PA with or without a preinjection of GST-RAP. Seven minutes after an intravenous injection of ^{125}I-rscu-PA with (open bars) or without (closed bars) a 1 min preinjection of 50 mg/kg body weight GST-RAP the amount of radioactivity in the various organs was determined. Recovery of the injected dose in the tissues shown was 56 ± 3 % for rscu-PA and 66 ± 4 % for rscu-PA with the preinjection of GST-RAP. Data represent the means of two experiments ± the variation of the individual experiments.

Injection of GST-RAP (50 mg/kg b.w.) 1 min prior to ^{125}I-rscu-PA reduced the liver uptake 1.3-fold and concurrently the amount of ^{125}I-rscu-PA in the serum increased 1.3-fold. The uptake in kidneys, lungs and muscles was not significantly affected, while the uptake of rscu-PA in the skin was slightly increased.

Effect of GST-RAP on the serum decay, liver uptake and liver cell distribution of ^{125}I-delta125-rscu-PA

To determine the role of residues 11-135 in the *in vivo* interaction of rscu-PA with LRP we injected ^{125}I-delta125-rscu-PA and determined the serum decay and liver uptake (figure 5). The maximal liver uptake at 5 min after injection was 17.4 ± 0.3 %, which is 65 % lower than the liver uptake of ^{125}I-rscu-PA. The clearance of ^{125}I-delta125-rscu-PA was 0.97 ± 0.04 ml/min (table I), with a serum half-life of 1.34 ± 0.01 min for the α-phase and 37.72 ± 6.03 min for the ß-phase. ^{125}I-delta125-rscu-PA was removed 2.4-fold more slowly from the circulation than ^{125}I-rscu-PA. Preinjection of 50 mg GST-RAP/kg b.w. reduced the liver uptake of ^{125}I-delta125-rscu-PA to 3 % of the injected dose (figure 5) and the clearance of ^{125}I-delta125-rscu-PA was delayed to 0.79 ± 0.03 ml/min (table I).

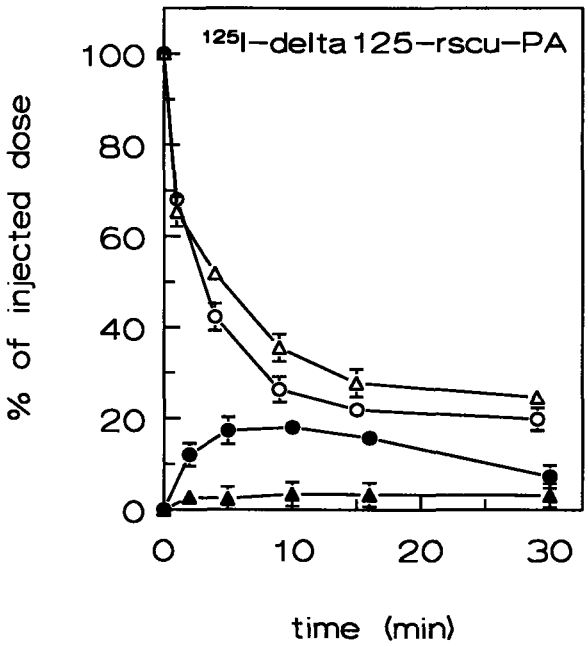

Figure 5. Inhibitory effect of GST-RAP on the serum decay and liver uptake of ^{125}I-delta125-rscu-PA. Rats were injected intravenously with (triangles) or without (circles) 50 mg/kg body weight GST-RAP 1 min prior to injection of 3 μg/kg body weight ^{125}I-delta125-rscu-PA. At the indicated times after injection of the radiolabelled ligand, liver uptake (closed symbols) and serum decay (open symbols) were determined. Data expressed as % of the injected dose represent the means of two experiments ± the variation of the individual experiments.

The uptake of delta125-rscu-PA in liver parenchymal, endothelial and Kupffer cells was determined and the highest specific association was found with the Kupffer cells (76.2 ± 11.9 % i.d. x 10^3/mg cell protein, (figure 2)). The specific association of delta125-rscu-PA with the Kupffer cells was 20-fold higher than with the parenchymal cells (3.7 ± 0.2 % i.d. x 10^3/mg cell protein). The parenchymal, Kupffer and endothelial cells contributed 60.2 %, 32.9 % and 7.0 % to the total liver uptake of delta125-rscu-PA, respectively. Compared to intact rscu-PA, the relative contribution of the Kupffer cells in the uptake of delta125-rscu-PA is decreased.

In vitro interaction of rscu-PA with isolated Kupffer cells

As has been mentioned, the Kupffer cells were responsible for 49.6 ± 4.0 % of the liver uptake of rscu-PA. The preinjection of GST-RAP resulted in a 62 % reduction in Kupffer cell association of rscu-PA, which may indicate a role of LRP in the recognition of rscu-PA. The presence of LRP on the Kupffer cells was confirmed by the displacement of ^{125}I-α_2-M-T by unlabelled GST-RAP (figure 6) with a K_i of 7.7 nM.

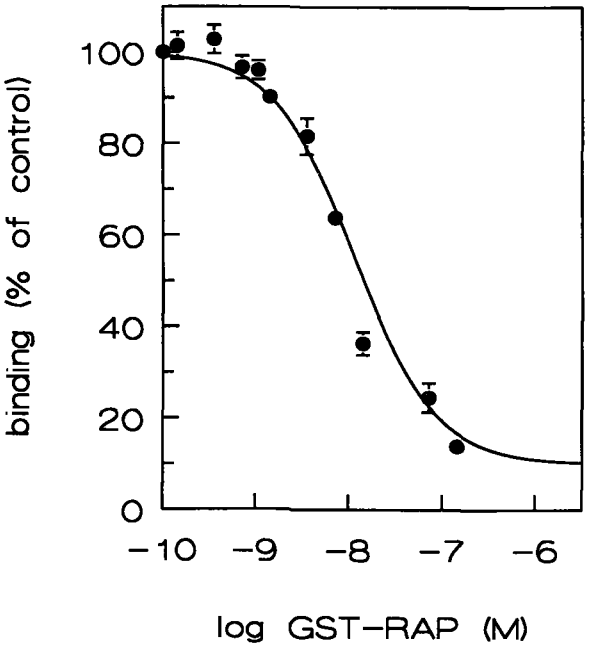

Figure 6. Recognition of ^{125}I-α_2-M-T by LRP on Kupffer cells. Freshly isolated rat Kupffer cells were incubated for 2 h at 4 °C in the presence of 4 nM ^{125}I-α_2-M-T with increasing concentrations of unlabelled GST-RAP. Data are expressed as % of control binding and represent the mean of 2 experiments ± the variation of the individual experiments.

Since delta125-rscu-PA was only recognized by LRP on the parenchymal- and Kupffer cells (no liver uptake when GST-RAP is injected) the remaining 38 %

Kupffer cell recognition of rscu-PA is likely to be mediated via residues 11-135. To investigate this interaction, freshly isolated Kupffer cells were incubated with phosphatidylinositol phospholipase C (PI-PLC) to remove glycosyl-phosphatidyl inositol (GPI)-linked proteins like uPAR. Table II shows that compared to the control situation the binding of ^{125}I-rscu-PA was reduced by 40 % after treatment of Kupffer cells with PI-PLC. The binding of ^{125}I-delta125-rscu-PA was not affected by the pretreatment of the cells with PI-PLC.

Table II: Recognition of ^{125}I-rscu-PA and ^{125}I-delta125-rscu-PA by Kupffer cells.

ligand	control (fmol/mg cell protein)	+ PI-PLC (fmol/mg cell protein)
rscu-PA	174.0 ± 36.4 (100 %)	103.0 ± 5.9 (59.2 ± 3.4 %)
delta125-rscu-PA	50.1 ± 7.5 (100 %)	50.8 ± 4.0 (101.4 ± 8.1 %)

Rat Kupffer cells were incubated for 2 h at 37 °C with or without 3 U/ml PI-PLC. After removal of the enzyme by washing, the cells were incubated for 2 h at 4 °C with 1 nM ^{125}I-rscu-PA or 1 nM ^{125}I-delta125-rscu-PA. The amount of cell associated radioactivity was determined. Binding is expressed as fmol/mg cell protein and represents the mean of 3 experiments ± S.D.

DISCUSSION

Urokinase is an attractive thrombolytic agent since it can activate the zymogen plasminogen to plasmin resulting in degradation of fibrin. As a consequence of a limited supply from natural sources and DNA technology thrombolytic therapy with recombinant u-PA is stimulated. u-PA is used as a fibrinolytic agent in the treatment of acute myocardial infarction.[12] A drawback of the use of u-PA as thrombolytic agent is its rapid clearance from the circulation[13,14], which is predominantly due to uptake by the liver.[13-18] Recently we have shown that the asialoglycoprotein receptor and LRP are hepatic receptors for natural glycosylated and *E.coli* produced recombinant u-PA, respectively.[18,19] In this study we investigated *in vivo* the role of LRP in the uptake of rscu-PA.

The 39-kDa RAP, produced as a fusion protein of GST and RAP, is a potent inhibitor of the binding of all known ligands of LRP. Since α_2-M-T is exclusively recognized by LRP on the liver parenchymal cells[36], we injected in rats 5, 15 or 50 mg GST-RAP/kg body weight (b.w.) prior to administration of ^{125}I-α_2-M-T in order to study the actual capacity of GST-RAP to block plasma clearance and liver uptake of ^{125}I-α_2-M-T via LRP. It was shown that GST-RAP inhibits the liver uptake

and serum clearance of ^{125}I-α_2-M-T in a concentration dependent way. An amount of 50 mg GST-RAP/kg b.w. was necessary to completely block the liver uptake and serum clearance of α_2-M-T. Therefore, we used a preinjection of 50 mg GST-RAP/kg b.w. to block LRP *in vivo* and determined its effect on the clearance and liver uptake of rscu-PA. It was shown that both the clearance and liver uptake of rscu-PA were reduced 1.5 and 1.6-fold, respectively. The reduction in liver uptake after GST-RAP preinjection was caused by a 91 % and 62 % reduction in the uptake of rscu-PA by parenchymal and Kupffer cells, respectively.

The difference in the capacity of GST-RAP to inhibit the clearance of α_2-M-T (a 5.1-fold) compared to rscu-PA (a 1.5-fold) may be explained by the difference in recognition of both ligands. α_2-M-T is exclusively recognized by LRP on the parenchymal liver cells and not by any other site in the body.[50] As we show now rscu-PA is also taken up by other organs and non-LRP sites in the liver. Therefore, a preinjection of GST-RAP, blocking the LRP mediated liver uptake of α_2-M-T and rscu-PA, will have a more dramatic effect on the clearance of α_2-M-T than on the clearance of rscu-PA.

Because of the high dose of GST-RAP used *in vivo*, other GST-RAP binding receptors such as the VLDL receptor (VLDLr), gp330 and the LDL receptor (LDLr) may also be inhibited. On isolated parenchymal cells however, GST-RAP inhibits the binding of rscu-PA with a K_d = 1.1 nM[19] which is indicative for an inhibition of LRP by GST-RAP, since the affinity of GST-RAP for the LDLr is about 100-fold lower. Therefore we conclude that also *in vivo* LRP mediates the uptake of rscu-PA by the liver cells and not the LDLr, which is also present on liver cells. RAP is not a suitable clinical candidate for the specific inhibition of the interaction of rscu-PA during thrombolytic therapy, because of the multiple ligand recognition capacity of LRP and binding of RAP to the other members of the LDL-receptor family. Blockage of LRP with RAP will inevitability lead to unwanted side effects. On the basis of an analysis of the specific moiety and/or amino acids within rscu-PA which interact with LRP, the development of long circulating rscu-PA can however be initiated.

The 60 % reduction in Kupffer cell uptake by GST-RAP indicates that LRP is at least partially responsible for the binding of rscu-PA to Kupffer cells. The presence of LRP on these cells was confirmed by the finding that the binding of ^{125}I-α_2-M-T to freshly isolated Kupffer cells could be inhibited completely by GST-RAP with a K_i of 7.7 nM, which is comparable to the K_i for the displacement of ^{125}I-α_2-M-T from parenchymal cells by GST-RAP (personal observation). The presence of LRP on Kupffer cells is in accordance with immunohistochemical studies of Zheng *et al.*[51] Besides the liver, the kidneys, lungs, muscles and skin contributed to the clearance of rscu-PA but the uptake in these organs was not inhibited by GST-RAP, indicating that LRP (and the other RAP-binding receptors) did not play a role in the limited uptake of rscu-PA in these organs. The liver is

the main organ for the uptake of GST-RAP. Already 2 minutes after injection about 80 % of the injected dose is present in the liver, even when a higher dose (100 mg/kg) of GST-RAP is used (personal observation). Hardly any GST-RAP is taken up by other organs. Since rscu-PA is taken up by other organs and GST-RAP not, we may conclude that rscu-PA does not bind to a member of the LDL receptor family in these organs.

In order to investigate the moiety in rscu-PA that accounted for the interaction with LRP *in vivo*, experiments were performed with a mutant of rscu-PA lacking residues 11-135. When injected into rats ^{125}I-delta125-rscu-PA was removed from the circulation at a 2.4 times slower clearance rate than rscu-PA and showed a 3-fold reduced liver uptake. GST-RAP completely reduced the liver uptake of delta125-rscu-PA indicating that LRP is likely to mediate its hepatic uptake. This is in accordance with the multipoint attachment model for binding of scu-PA or u-PA:PAI-1 to LRP as postulated by Nykjaer *et al.*[52] They showed that both the amino terminal fragment (ATF) and the serine protease domain of scu-PA are important for the interaction with purified LRP. In their model deletion of the ATF domain resulted in a decreased affinity for LRP. In our system, deletion of the ATF domain resulted in a reduction in liver uptake.

Although the liver uptake of delta125-rscu-PA was completely blocked by GST-RAP still a relatively rapid clearance from the circulation was observed, probably due to extra-hepatic uptake of this rather small molecule (31.8 kDa). The latter is in line with Breton *et al.*[53] who showed that a conjugate of delta125-rscu-PA with serum albumin injected in monkeys shows a dramatic increase in serum half-life compared to delta125-rscu-PA alone. It is thought that the conjugation with albumin protects the delta125-rscu-PA molecule probably for extravascularization.

In addition to LRP, uPAR may contribute to the liver uptake of rscu-PA. Pretreatment of the Kupffer cells with PI-PLC, removing GPI-membrane bound proteins like uPAR, reduced the Kupffer cell association of intact rscu-PA by 40 %, while the association of delta125-rscu-PA was not affected. This is in accordance with recognition of scu-PA by uPAR, since u-PA is recognized by this receptor via the epidermal growth factor domain in which especially residue 12-32 are directly implicated in the binding.[54] These residues are not present in delta125-rscu-PA. Other evidence for a role of uPAR in the liver uptake *in vivo* is that the liver association of ^{125}I-rscu-PA in the situation when LRP was blocked by GST-RAP remained at a steady level up to 30 min after injection. This may point to recognition by a non-internalizing receptor.

Species specificity has been described for the recognition of human u-PA by murine uPAR and reverse. This is based upon differences in the amino acid composition of the receptor binding sequence (residue 12-32) of u-PA.[55] Furthermore, it is also shown by Rabbani *et al.*[56] that the rat uPAR domain I, that is responsible for the interaction with u-PA, shows an amino acid homology of

only 57 % with the corresponding human uPAR domain I sequence. Both differences in amino acid composition of the receptor binding sequence of u-PA and in the ligand recognition domain of uPAR may be responsible for the species specificity. This species specificity may also play a role in our system. Human rscu-PA shows *in vitro* a low affinity for rat uPAR , but *in vivo* the species specificity may be less stringent. In man, the Kupffer cell recognition of (human) rscu-PA may be more important.

The possibility that u-PA is not taken up by the liver as scu-PA but as a tcu-PA:PAI-1 complex is unlikely since, the formation of complexes between u-PA and its inhibitors is mostly thought to be preceded by the activation of scu-PA to tcu-PA. Scu-PA is stable in plasma for more than 72 hours at 37 °C (< 2.5 μg/ml[57]) and it does not form SDS-stable complexes. Since we used low doses of rscu-PA (about 50 ng/ml) and rscu-PA is rapidly cleared by the liver, we conclude that it is most likely that rscu-PA reaches the liver in an uncomplexed form. Furthermore, isolated parenchymal and Kupffer cells do not produce PAI-1[58,59], but still scu-PA is degraded by these cells. From the *in vitro* data we conclude that it is at least not necessary that u-PA:PAI-1 complexes are formed for an adequate degradation of u-PA by liver cells.

In conclusion, our findings show that *in vivo* rscu-PA is recognized by the rat parenchymal liver cells mainly by LRP. LRP is also responsible for 60 % of the uptake of rscu-PA by the Kupffer cells. For the residual 40 % Kupffer cell binding of rscu-PA uPAR is involved. With this knowledge it should be possible to develop specific inhibitor(s) for LRP and uPAR resulting in a prolongation of the half-life of rscu-PA during thrombolytic therapy and/or to design more specific variant forms of rscu-PA with extended blood circulation times.

ACKNOWLEDGEMENTS

We thank Dr. J. Herz (Dallas, Texas, USA) for providing us with the pGEX-RAP plasmid and Dr. A. Molinari (Farmitalia Carlo Erba, Milan, Italy) for providing us with the rscu-PA and delta125-rscu-PA.
This work was supported by Grant 92.139 from the Netherlands Heart Foundation.

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Chapter 4

Clearance of urinary urokinase-type plasminogen activator by the asialoglycoprotein receptor and proteoglycans on rat parenchymal liver cells

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SUMMARY

In this study we determined by means of receptor isolation and *in vivo* as well as *in vitro* experiments which receptors on rat parenchymal liver cells are involved in the binding and uptake of urinary urokinase-type Plasminogen Activator (tcu-PA). Isolation of the binding protein from rat and human liver using a tcu-PA-Sepharose 4B column followed by immunoblotting demonstrated that a protein that bound to urinary tcu-PA was the asialoglycoprotein receptor (ASGPr). In rats, ^{125}I-tcu-PA was rapidly cleared from the circulation ($t_{1/2}$ = 1 min, clearance 1.6 ml/min). The liver uptake was maximally 40 % of the injected dose. An excess of unlabelled asialo-orosomucoid or enzymatic removal of the carbohydrate moiety of ^{125}I-tcu-PA resulted in a 42 % reduction of the liver uptake and a slightly increased plasma half-life. Inhibition of the interaction of tcu-PA with heparan sulfate proteoglycans (HSPGs) by the injection of protamine sulfate resulted in a 14 % decrease in liver uptake and a 1.7-fold delay in serum clearance (clearance 0.9 ml/min). Combined blockade of ASGPr and HSPGs resulted in a 58 % reduced liver uptake and a 2-fold delay in serum clearance (0.8 ml/min). Within the liver, the parenchymal cells were responsible for 95 % of the tcu-PA uptake. Besides the liver, kidneys (6.4 %), skin (6.1 %) and muscles (4.4 %) contributed to the clearance of tcu-PA. *In vitro* studies with freshly isolated parenchymal liver cells showed that tcu-PA bound to the cells with a K_i of 103 nM. Binding could be inhibited for 30 % with protamine sulfate and for 80 % with D-galactose (K_i = 15.3 mM) or asialo-orosomucoid (K_i = 3.0 nM) or an antibody against the ASGPr.

It is concluded that the asialoglycoprotein receptor and heparan sulfate proteoglycans on the rat parenchymal liver cells are involved in the rapid plasma clearance and liver uptake of urinary tcu-PA.

INTRODUCTION

Urokinase-type plasminogen activator (u-PA) is a highly specific serine protease that converts plasminogen to plasmin. Plasmin is a broad spectrum protease that degrades fibrin and several proteins of the extracellular matrix. Because of its capacity to degrade fibrin via plasminogen activation, u-PA is an attractive thrombolytic agent. The u-PA preparations that are commercially available and used in the clinic are currently extracted from urine.

Urokinase is produced as a single-chain protein (scu-PA) with a molecular mass of 55,000 (= high molecular weight scu-PA (HMW-scu-PA)) by e.g. cultured kidney cells[1,2], tumor cells[3] and fibroblasts.[4] Proteolytic cleavage of scu-PA between Lys_{158} and Ile_{159} by plasmin or kallikrein results in the conversion into an active two-chain form (tcu-PA). A low molecular weight form of scu-PA (LMW-scu-PA), lacking the first 143 N-terminal amino acids, has also been isolated.[2,5] When

expressed in eukaryotic cells, u-PA is secreted as a glycoprotein with a single N-linked oligosaccharide at Asn_{302}.[6]

A major drawback of the use of u-PA as thrombolytic agent is its rapid clearance from the circulation.[7,8] The liver and to a lesser extent the kidneys have been identified to play a major role in the uptake of HMW- and LMW-u-PA in monkeys, rabbits and rats.[5,7-10] Three cellular recognition sites have been described for u-PA. One receptor is present on e.g. human monocytes and related tumor cells, smooth muscle cells, fibroblasts, endothelial cells and keratinocytes.[11-13] This receptor, termed uPAR, is a heavily glycosylated protein with a molecular mass of 55,000-60,000[13,14], which is attached to the cell membrane via a glycosyl phosphadityl inositol (GPI) anchor.[15] It recognizes the epidermal growth factor domain (residue 5-45) in u-PA.[16]

The second recognition site for u-PA and for a complex of u-PA with Plasminogen Activator Inhibitor-1 (PAI-1) is the Low-Density Lipoprotein Receptor-related Protein/α_2-Macroglobulin Receptor (LRP/α_2-MR).[17-20] LRP is a 600 kDa cell surface receptor that is expressed by a broad spectrum of cell types, e.g. hepatocytes, macrophages and fibroblasts.[21-24] A 39-kDa Receptor-Associated Protein (RAP) is non-covalently associated with LRP. LRP binds in addition to u-PA several apparently unrelated ligands: RAP, α_2-M-proteinase complexes[21], apoE enriched ß-migrating Very-Low Density Lipoprotein (ß-VLDL)[25], lipoprotein lipase[26], tissue-type plasminogen activator (t-PA)[27], t-PA:PAI-1 and u-PA:PAI-1 complexes.[17-20,28] Binding of all these ligands to LRP can be blocked by RAP.

Thirdly, we recently described that *E.coli* produced recombinant scu-PA is bound and degraded by rat parenchymal liver cells via LRP, whereas HMW-scu-PA produced by cultured kidney cells is recognized by a high affinity lectin-like recognition site on the parenchymal liver cells.[20] The aim of the present study was to establish by means of receptor isolation and *in vivo* and *in vitro* experiments the identity of the recognition site which is responsible for the rapid liver uptake of urinary tcu-PA.

MATERIALS AND METHODS

Materials

Phenylmethylsulphonyl fluoride ($PhMeSO_2F$), goat anti-rabbit IgG antibody conjugated with alkaline phosphatase, human orosomucoid, neuraminidase (agarose bound), protamine sulfate, D-galactose, bovine serum albumin (BSA, fraction V), collagenase type I and type IV, heparinase type I and chondroitin lyase ABC were from Sigma (St. Louis, MO, USA). Triton X-100 and 2-(4-(-hydroxyethyl)-1-piperazinyl)-ethanesulfonic acid (Hepes) were from Merck (Darmstadt, Germany). N-glycosidase F and pronase were from Boehringer Mannheim (Mannheim, Germany). CNBr-activated Sepharose 4B and Sepharose CL-6B were from Pharmacia-LKB (Uppsala, Sweden). Two-chain urinary urokinase (tcu-PA, "Ukidan") was from Serono (Aubonne, Switzerland). Rabbit antiserum against the total rat asialoglycoprotein receptor recognizing all subunits was kindly donated by Dr. K. Drickamer (New York, USA). The IgG fractions of this antiserum and non-

immune rabbit serum were isolated using Protein A Sepharose chromatography (Pharmacia-LKB). GST-RAP, a conjugate of the receptor associated protein and glutathione S-transferase[29] was produced in *E.coli*[30] and showed similar inhibitor characteristics as RAP. The pGEX-RAP plasmid was a generous gift from Dr. J. Herz (Dallas, Texas, USA). Dulbecco's modified Eagle medium (DMEM) was from Gibco (Irvine, Scotland). ^{125}I (carrier free) in NaOH was obtained from Amersham (Buckinghamshire, United Kingdom). All other chemicals were of analytical grade.

Radiolabeling of tcu-PA and ASOR

Tcu-PA and asialo-orosomucoid (ASOR) were iodinated as described earlier[10] using the iodogen method, resulting in a specific radioactivity of 3000-3500 cpm/ng and 680 cpm/ng respectively.

Isolation of binding protein(s) from liver tissue at 4 °C

Rat livers were isolated from male Wistar rats, while human liver tissue was obtained at autopsy. Sixty grams of rat or human liver tissue was homogenized in a blender with 600 ml dissociation buffer (50 mM Tris-HCl, pH 7.4, containing 1.25 M NaCl, 15 mM EDTA and 1 mM $PhMeSO_2F$) and stirred for 30 min. The homogenate was centrifuged at 40,000 xg and the insoluble fraction was washed twice with 300 ml dissociation buffer and twice with 300 ml loading buffer (50 mM Tris-HCl, pH 7.4, containing 1.25 M NaCl, 15 mM $CaCl_2$ and 1 mM $PhMeSO_2F$). The final pellet was extracted overnight with 150 ml loading buffer supplemented with 1% Triton X-100. The extract was centrifuged and stored at -70 °C.

Sixty ml of extract was diluted 2-fold (or 10-fold when indicated) with loading buffer/1 % Triton X-100 and applied to a 2 ml tcu-PA-Sepharose 4B column (containing 5 mg urokinase/ml CNBr-activated Sepharose 4B). The column was washed with loading buffer/1 % Triton X-100 and with pre-elution buffer (50 mM Tris-HCl, pH 7.4, containing 0.1 M NaCl, 3 mM $CaCl_2$, 1 mM $PhMeSO_2F$ and 0.1 % Triton X-100). Elution was performed with the latter buffer containing 3 mM EDTA instead of 3 mM $CaCl_2$ (elution buffer). When indicated, elution was carried out with pre-elution buffer supplemented with 0.1 M D-galactose or with 40 mM ammonium acetate, pH 6.0, containing 0.1 M KCl, 0.1 % Triton X-100 and 0.1 % Tween 80.[31] The extracts were also applied to D-galactose-Sepharose CL-6B (6 ml column, containing 120 mg/ml D-galactose) or asialo-orosomucoid-Sepharose 4B (1 ml column, containing 2.4 mg/ml immobilized glycoprotein). D-galactose was coupled to Sepharose CL-6B by using the divinyl sulphone method.[32] Asialo-orosomucoid was prepared by treating orosomucoid with neuraminidase-agarose.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to Laemmli[33] using 10% gels. One hundred μl of protein sample was lyophilized and dissolved in SDS-sample buffer. To the protein preparations that did not contain EDTA (for instance those eluted with D-galactose or with ammonium acetate buffer pH 6.0) 10 mM EDTA was added, which resulted in sharper bands on SDS gels. Silver staining was performed according to Morrissey.[34]

Immunoblots on nitrocellulose were developed by using 50 μg/ml rabbit anti-rat ASGP-receptor IgG or non-immune rabbit IgG as a control, and goat anti-rabbit IgG antibody conjugated with alkaline phosphatase, and stained according to Blake *et al.*[35]

Treatment of ^{125}I-tcu-PA with N-glycosidase F

Radiolabelled tcu-PA (10 μg) was treated for 18 h at 37 °C with 4 U/ml N-glycosidase F in 50 mM phosphate buffer, 10 mM EDTA, 10 mM NaN_3, pH 7.2, followed by dialysis against PBS. Removal of the carbohydrate moiety was checked by 10 % SDS-PAGE.

Serum decay and tissue uptake

Male Wistar rats (200-250 g) were anaesthetized by intraperitoneal injection of 15-20 mg of sodium pentobarbital. The abdomen was opened, radiolabelled ASOR (6 μg/kg rat) or tcu-PA (800 ng/kg rat) inactivated by incubation for 10 min at room temperature with 2 μM of GGACK, was

injected via the inferior vena cava and at the indicated times 0.3 ml blood samples were taken from the inferior vena cava and liver lobules were tied off. The body temperature of the rats was kept at 36.5-37 °C using an infrared lamp. The respiration was facilitated by administration of carbogen 95/5 (95 % O_2, 5 % CO_2). After clotting, blood samples were centrifuged for 2 min at 10,000 xg and 100 µl serum samples were counted for radioactivity. The excised liver lobules (totally less than 15 % of the total liver mass) were weighed and radioactivity was counted. Total liver uptake was determined by weighing the liver at the end of the experiment. The total amount of radioactivity in the serum and liver lobules was determined as described previously.[10,36,37]

Uptake of ^{125}I-tcu-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 present in these organs.[10]

Liver cell distribution of tcu-PA

Rats were anaesthetized and injected with 800 ng of GGACK-treated ^{125}I-tcu-PA. Parenchymal, endothelial and Kupffer cells were separated by collagenase perfusion (collagenase type I), followed by differential centrifugation and counterflow centrifugal elutriation at 4 °C as described in detail elsewhere.[38-41] The contributions of the different liver cell types to the total hepatic uptake of the injected ligand were calculated with the assumption that parenchymal, endothelial and Kupffer cells account for 92.5, 3.3 and 2.5% of the total liver protein mass, respectively.[38-41] As found for a number of substrates[38-41], 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.

Isolation of parenchymal liver cells

Male Wistar rats (250-300 g) were anaesthetized and parenchymal liver cells were isolated by perfusion of the liver for 9 min with collagenase (type IV, 0.05% (w/v)) at 37 °C by the method of Seglen[42], modified as described previously.[43] The obtained parenchymal liver cells were ≥ 95% viable as judged by 0.2 % trypan blue exclusion and ≥ 99 % pure as judged by light microscopy. For binding studies the parenchymal liver cells were resuspended in DMEM supplemented with 2 % BSA (pH 7.4).

Treatment of cells with heparinase I and chondroitin lyase ABC

Isolated rat parenchymal liver cells (approx. 16 mg of cell protein) were incubated for 40 min at 37 °C in the presence of heparinase I (2.4 U/ml) or chondroitin lyase ABC (0.24 U/ml) in DMEM supplemented with 2 % BSA (pH 7.4) in a total volume of 4 ml.[44] Control cells were incubated without enzyme. After incubation the cells were centrifuged at 50 xg for 1 min at 4 °C and washed twice with DMEM containing 2 % BSA (pH 7.4). After incubation of the cells with the above mentioned enzymes, the viability of the enzyme-treated cells was not different from that of control cells. Enzyme treated and control cells were incubated for 2 h at 4 °C with 1 nM ^{125}I-tcu-PA.

In vitro binding of tcu-PA

Parenchymal cells (1-2 mg cell protein/ml DMEM 2% BSA) were incubated with 1 nM ^{125}I-tcu-PA or 5 nM ^{125}I-ASOR with or without competitors at the indicated concentrations in a volume of 0.5 ml DMEM containing 2 % (w/v) BSA pH 7.4. Incubations were carried out in plastic containers (Kartell, Milan, Italy) for 2 h at 4 °C and continuously shaken (150 rpm, Adolf Kuhner, Basel, Switzerland). At the end of the incubations cells were washed twice at 4 °C for 30 sec at 50 xg with buffer containing 10 mM Tris-HCl, 0.15 M NaCl, 5 mM $CaCl_2$, 0.2 % BSA, pH 7.4 (at 4 °C), followed by one wash with the same buffer without BSA. Finally, cells were resuspended in this buffer (without BSA) and cell bound radioactivity was counted in a gamma counter. The amount of cell protein was determined as described by Lowry *et al.*[45] with BSA as standard.

Pharmacokinetic analysis and statistics

Binding parameters (K_i) were determined as described before[20] from displacement curves using a sigmoid model of the binding data with a 'Hill-slope' that varied from 0.9-1.0 representing a one-site binding model (Graphpad Prism: 1994, GraphPad Software, Inc., San Diego, U.S.A.). Plasma clearance curves were analyzed by computerized nonlinear fitting following a biphasic clearance model using the same program ($t_{½\alpha}$, $t_{½ß}$, AUC_{0-29}). The clearance was calculated by the formula: clearance = dose/AUC_{0-29}. Statistical analysis of the clearance values (unpaired, two-sided students t-test) was performed by using the Instat program of Graphpad Prism.

RESULTS

Isolation of binding protein(s) on a tcu-PA-Sepharose 4B column

A 1 % Triton X-100 solubilisate of rat liver tissue was applied to a tcu-PA-Sepharose 4B column. The column was eluted using a buffer containing 3 mM EDTA. The protein composition of the eluate was analysed by SDS-PAGE (figure 1).

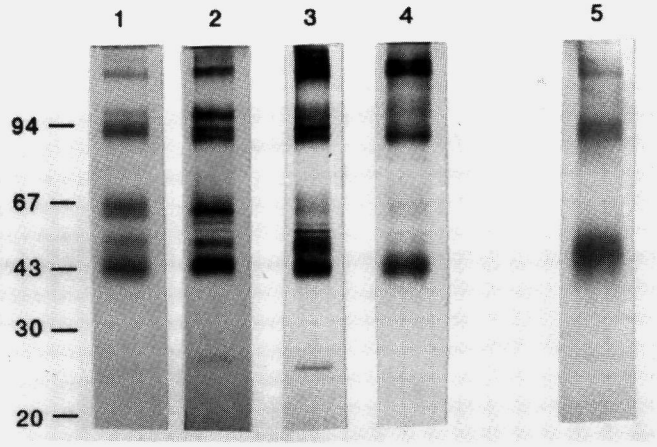

Figure 1. SDS-PAGE of the purified protein preparations by tcu-PA-Sepharose chromatography. SDS-polyacrylamide gel electrophoresis of protein preparations obtained by tcu-PA-Sepharose chromatography of non-reduced Triton X-100 extracts of rat (lanes 1-4) and human (lane 5) liver. Lane 1-4 shows four typical examples out of ten independent isolations. The extracts were diluted 2-fold (lanes 1, 2 and 5) or 10-fold (lanes 3 and 4) with loading buffer/1% Triton X-100 before applying them to the column. The bound proteins were eluted with 3 mM EDTA (lanes 1-3 and 5) or pH 6.0 buffer (lane 4); see Materials and Methods for details. The positions of the following calibration proteins are indicated: phosphorylase b (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa) and trypsin inhibitor (20.1 kDa).

This procedure was carried out ten times during a period of one year. Apart from some small differences, the results were nearly identical. Figure 1 (lanes 1-3) shows three typical examples. Major protein bands were observed in three areas: between 40 and 60 kDa, between 90 and 110 kDa and above 150 kDa. The 40-60 kDa area contained always a main band at 44 kDa and two weaker bands at 49 and 60 kDa. The latter band was less pronounced when the extract was diluted 10-fold before application of the liver solubilisate to the column (figure 1, lanes 3 and 4), and may contain some non-specifically bound albumin. The two other areas at 90-110 kDa and above 150 kDa contained each two or three closely spaced protein bands.

The results were very similar after elution of the tcu-PA-Sepharose column with a slightly acidic buffer (pH 6.0, figure 1, lane 4) or with a D-galactose (0.1 M) containing buffer instead of EDTA. A similar protein pattern was obtained when the liver solubilisate was applied to a D-galactose-Sepharose or asialo-orosomucoid-Sepharose column instead of the tcu-PA-Sepharose column (not shown). These results indicate that the solubilized liver proteins interacted with tcu-PA-Sepharose in a galactose-dependent manner and may be related to the well-known asialoglycoprotein receptor (ASGPr).

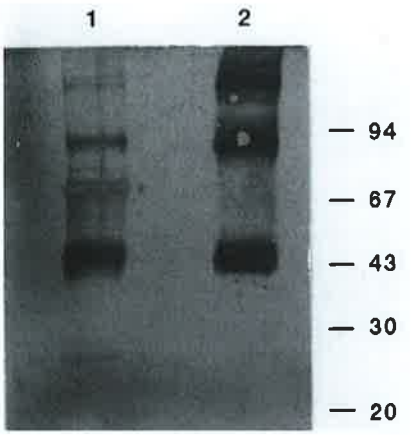

Figure 2. Immunoblot of partially purified rat liver binding protein(s). The protein preparation shown in figure 1 (lane 4) was subjected to SDS-PAGE, blotted onto nitrocellulose and stained by using antibodies against rat asialoglycoprotein receptor. Controls with non-immune IgG were negative (not shown). Lane 1, reduced; lane 2, non-reduced. Calibration protein as in figure 1 (non-reduced).

Figure 2 (lane 2) shows that all major proteins isolated on the tcu-PA-Sepharose column cross-reacted with a monospecific polyclonal antibody against rat ASGPr. After reduction with ß-mercaptoethanol most of the protein bands with a molecular mass between 90-110 kDa and more than 150 kDa disappeared and only 40-60 kDa proteins that reacted with the anti-ASGPr antiserum, were identified (figure 2, lane 1).

In addition to rat liver tissue, human liver tissue was extracted with 1 % Triton X-100 and applied to the tcu-PA-Sepharose column. Elution with EDTA (3 mM) resulted in a protein pattern on SDS-PAGE similar to that obtained with rat liver tissue (figure 1, lane 5). Human liver proteins eluted from the tcu-PA-column were applied to an asialo-orosomucoid-Sepharose column. It was determined that the three major proteins bound in a calcium-dependent manner to the asialo-orosomucoid column (not shown).

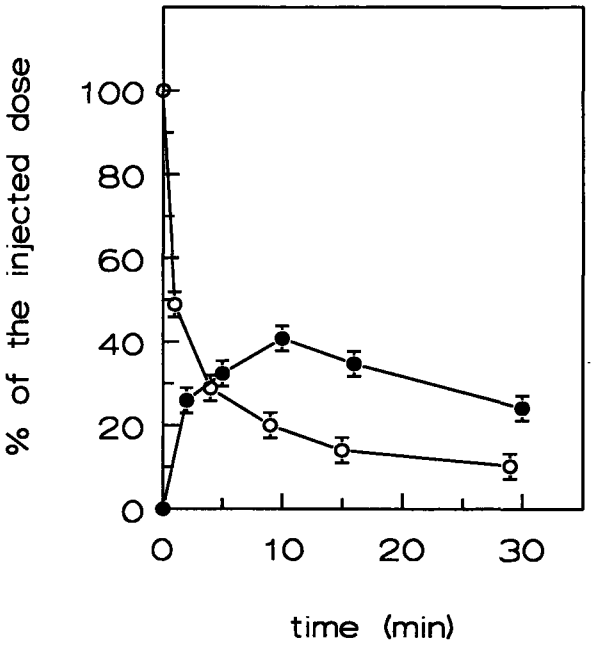

Figure 3. Serum decay and liver uptake of ^{125}I-tcu-PA. Rats were injected intravenously with ^{125}I-tcu-PA (800 ng/kg rat) which was inactivated by a preincubation for 10 min with GGACK. At the indicated times, radioactivity in serum (○) and liver lobules (●) were determined. Data expressed as % of the injected dose are mean (n = 3) ± S.D.

Serum decay, liver uptake and organ distribution of tcu-PA: effect of inhibitors

^{125}I-tcu-PA was inactivated with 2 μM GGACK (GGACK-treated ^{125}I-tcu-PA) in order to prevent binding to PAI-1. After intravenous injection of GGACK-treated ^{125}I-tcu-PA into rats a rapid decay from the blood circulation was observed (clearance = 1.6 ± 0.1 ml/min (table I)), that was accompanied by an uptake of ^{125}I-tcu-PA in the liver (figure 3). The maximal liver uptake was observed at 10 min after injection (40.8 ± 1.6 % of the injected dose). Between 15 and 30 min after injection, the liver-associated radioactivity decreased from 41 to 24 %, most likely due to degradation of tcu-PA.

Table I: Clearance values for the serum decay of ^{125}I-tcu-PA with and without competitors.

ligand	clearance (ml/min)
tcu-PA	1.6 ± 0.1 (n=4)
N-glycosidase F treated tcu-PA	1.2 ± 0.1 (n=3)**
tcu-PA + ASOR	1.2 ± 0.2 (n=4)*
tcu-PA + GST-RAP	1.4 ± 0.2 (n=3)^{ns}
tcu-PA + protamine sulfate	0.9 ± 0.1 (n=3)***
tcu-PA + ASOR + protamine sulfate	0.8 ± 0.1 (n=3)***

Pharmacokinetic parameters were derived from computer fitting as described in material and methods. The clearance curves are shown in figure 4. Significance of the observed changes in clearance values compared to the tcu-PA control was tested by the unpaired, two-sided students t-test. (***): extremely significant ($p<0.001$), (**): very significant ($p<0.01$), (*): considered significant, (^{ns}): not significant.

In order to investigate the possible involvement of the ASGPr in the liver uptake of tcu-PA, an inhibiting amount of the ASGPr antagonist ASOR[46] (15 mg/kg rat) was injected 1 min prior to GGACK-treated ^{125}I-tcu-PA (figure 4). The maximal liver uptake at 10 min after injection was decreased from 40.8 ± 1.6 % to 23.7 ± 3.5 % of the injected dose. The clearance of GGACK-treated ^{125}I-tcu-PA was reduced 1.3-fold from 1.6 ± 0.1 ml/min for control rats to 1.2 ± 0.2 ml/min for rats pretreated with ASOR (table I).

In order to establish the role of the carbohydrate side chain of urokinase in its clearance, ^{125}I-tcu-PA was treated with N-glycosidase F for 18 hours at 37 °C, inactivated by GGACK, and injected intravenously into rats (figure 4). The liver uptake at 10 min after injection was lowered from 40.8 ± 1.6 % for control tcu-PA to 22.2 ± 1.1 % for N-glycosidase F treated tcu-PA. The clearance for N-glycosidase F treated ^{125}I-tcu-PA was reduced to same level (1.2 ± 0.1 ml/min) as the clearance in ASOR pretreated rats (table I).

GST-RAP (50 mg/kg rat)[47] was injected 1 min prior to injection of GGACK-treated ^{125}I-tcu-PA in order to determine the possible involvement of LRP in the recognition of tcu-PA *in vivo* (figure 4). No significant effect on the liver uptake and serum decay of tcu-PA was observed while the dose used is sufficient to completely inhibit the binding of α_2-macroglobulin to LRP.[47]

Tcu-PA shows a net positive charge, possesses a strong heparin binding site in its kringle domain[48] and may bind heparin via its protease domain.[49] This may result in the binding of tcu-PA to negatively charged proteoglycans on the liver cell surface. Protamine sulfate was used as a competitor for the binding to heparan sulfate proteoglycans (HSPGs) *in vivo*.[50] Protamine sulfate (50 mg/kg rat) was injected 1 min prior to GGACK-treated ^{125}I-tcu-PA (figure 4). Preinjection of protamine sulfate slightly reduced the liver uptake from 40.8 ± 1.6 % to 35.2 ± 1.6

% of the injected dose. Although the liver uptake was not reduced dramatically, the effect of protamine sulfate on the serum clearance of tcu-PA was significant. The clearance was reduced a 1.8-fold, from 1.6 ± 0.1 ml/min to 0.9 ± 0.1 ml/min (table I). When both ASOR (15 mg/kg rat) and protamine sulfate (50 mg/kg rat) were injected 1 min prior to injection of ^{125}I-tcu-PA the maximum liver uptake was reduced to 17 % of the injected dose (figure 4). The serum clearance was reduced 2-fold to 0.8 ± 0.1 ml/min (table I). As a control, the effect of the preinjection of protamine sulfate on the recognition of ^{125}I-ASOR by the asialoglycoprotein receptor *in vivo* was studied (not shown). No effect on liver uptake or serum decay of ^{125}I-ASOR was observed.

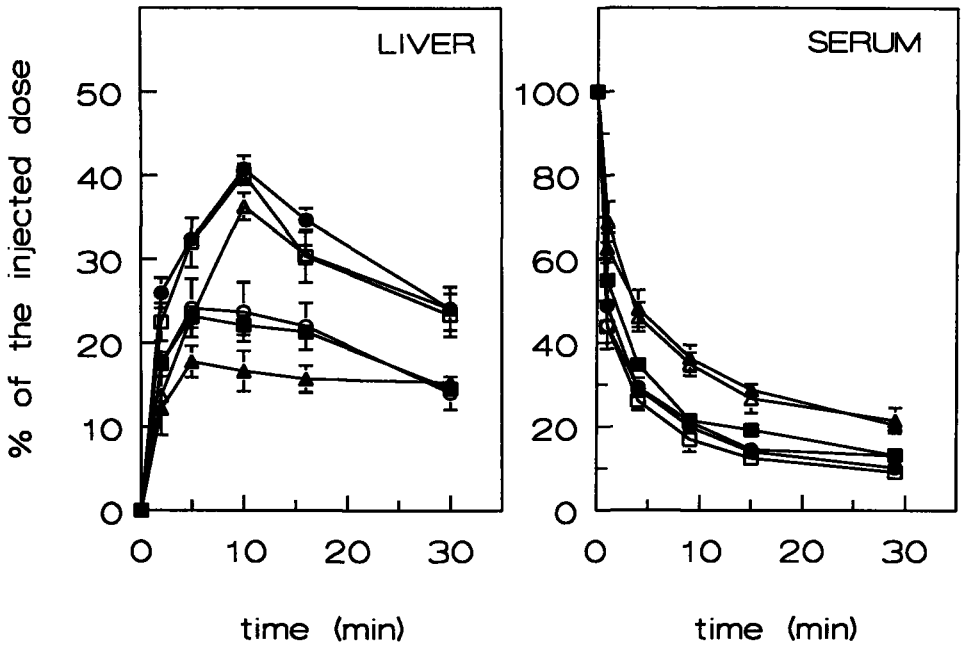

Figure 4. Effect N-glycosidase F treatment or a preinjection of ASOR, GST-RAP or protamine sulfate on the liver uptake and serum decay of ^{125}I-tcu-PA. Rats were injected intravenously with (○) or without (●) a preinjection of 15 mg/kg rat ASOR 1 min prior to injection of GGACK-treated ^{125}I-tcu-PA. With N-glycosidase F treated GGACK-treated ^{125}I-tcu-PA (■) or with a preinjection of 50 mg/kg body weight GST-RAP (□) 1 min prior to injection of GGACK-treated ^{125}I-tcu-PA. Other rats were injected with 50 mg/kg body weight protamine sulfate (Δ) or with ASOR and protamine sulfate (▲) 1 min prior to injection of GGACk-treated ^{125}I-tcu-PA. At the indicated times after injection of the radiolabelled ligands, the liver uptake and serum decay were determined. Data are expressed as % of the injected dose and represent the means of 3 or 4 experiments ± S.D.

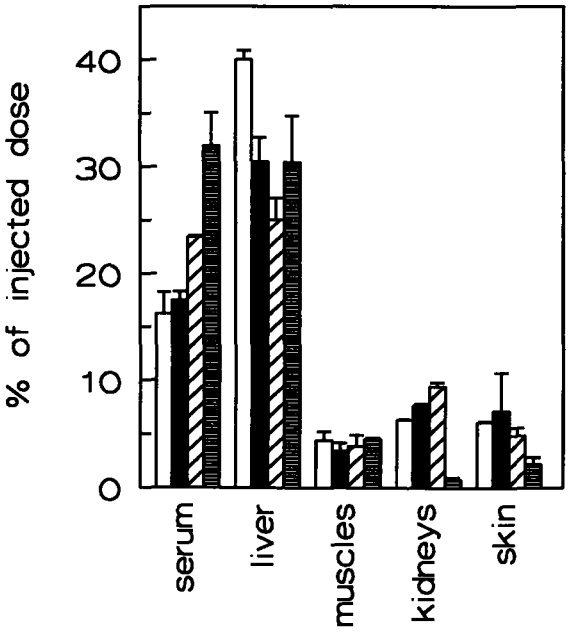

Figure 5. Tissue distribution of intravenously injected N-glycosidase F treated ^{125}I-tcu-PA or ^{125}I-tcu-PA with or without preinjection of ASOR or protamine sulfate. Ten min after an intravenous injection of the ligands as described in figure 4, the amount of radioactivity in the various organs was determined. Recovery of the injected amount of radioactivity (in the organs shown) was 73 ± 3 % for tcu-PA (open bars), 66 ± 1 % for tcu-PA with a preinjection of ASOR (closed bars), 67 ± 4 % for N-glycosidase F treated tcu-PA (diagonally striped bars) and 70 ± 2 % for tcu-PA with a preinjection of protamine sulfate (horizontally striped bars). Data expressed as % of the injected dose are mean (n = 3) ± S.D.

We determined whether other organs than the liver contributed to the removal of tcu-PA from the circulation (figure 5). Besides the liver (40.0 ± 0.9 %) the kidneys (6.4 ± 0.1 %), skin (6.1 ± 0.1 %) and muscles (4.4 ± 0.8 %) contributed to the serum decay. Other organs and tissues contributed each for less than 3 % to the uptake of tcu-PA. Injection of ASOR (15 mg/kg rat) 1 min prior to GGACK-treated ^{125}I-tcu-PA or treatment of ^{125}I-tcu-PA with N-glycosidase F reduced the uptake in the liver 1.5-fold (from 40 % to 26 % of the injected dose at 10 min after injection). Concurrently, the amount of ^{125}I-tcu-PA in the serum was increased 1.3-fold (from 16 % to 20 %), while the uptake in the kidneys tended to increase and the contribution of the other organs remained constant. Preinjection of protamine sulfate reduced the uptake of tcu-PA in the liver 1.3-fold (from 40 % to 30 % of the injected dose). The uptake by the kidneys was reduced 8-fold (from 6.4 % to 0.8 %) and the uptake by the skin was reduced a 2.8-fold (from 6.1 % to 2.2 %). The reduction in liver, kidneys and skin uptake was reflected in a 2-fold increase of ^{125}I-tcu-PA in the serum (from 16.2 % (control) to 32 % (protamine sulfate pretreated)).

Liver cell distribution of tcu-PA

In order to analyse the cellular recognition site of tcu-PA in the liver, we isolated parenchymal, endothelial and Kupffer cells at 10 min after injection of GGACK-treated ^{125}I-tcu-PA. Table II shows that the parenchymal cells were responsible for 94.5 % of the total liver uptake of tcu-PA. Non-parenchymal liver cells contributed only about 5 % to the total liver uptake of tcu-PA.

Table II. Relative contribution of the different liver cell types to the liver uptake of urinary tcu-PA.

Cell type	^{125}I-tcu-PA (%)
Parenchymal cells	94.5 ± 0.1
Endothelial cells	3.1 ± 0.7
Kupffer cells	2.4 ± 0.7

Different liver cells types were isolated 10 min after injection of GGACK-treated ^{125}I-tcu-PA. The % of the injected dose/mg cell protein in each cell fraction isolated (not shown) was multiplied by the relative contribution of each cell type to the total liver uptake. Recovery of the injected dose in the different cell types was 101.8 ± 4.3 %. Data represent the mean of 2 experiments ± the variation of the individual experiments.

In vitro interaction of tcu-PA with parenchymal liver cells

Since parenchymal liver cells were responsible for 95 % of the liver uptake, the specificity of the interaction of tcu-PA with this type of liver cells was studied *in vitro*. Displacement studies of ^{125}I-tcu-PA with unlabelled tcu-PA showed an apparent K_i for the binding of tcu-PA of 103.2 nM, with a non-specific binding of 20 % of the control (figure 6A). GST-RAP was up to 80 nM unable to compete for the binding of ^{125}I-tcu-PA to parenchymal liver cells. Protamine sulfate was able to displace about 30 % of the parenchymal liver cell binding of ^{125}I-tcu-PA (figure 6B). A K_i of 21.0 μM was calculated.

In order to identify the nature of the interaction of tcu-PA with the parenchymal liver cells competition experiments with D-galactose and ASOR were performed since the ASGPr was a likely candidate (figure 1-4). Figure 7 shows that the binding of ^{125}I-tcu-PA was inhibited by D-galactose (K_i = 15.3 mM) and by ASOR (K_i = 3.0 nM). The binding of tcu-PA could also be competed for by a monospecific antibody raised against the ASGPr (figure 7). Control rabbit IgG did not inhibit the binding of tcu-PA to parenchymal liver cells. As a control for the recognition of tcu-PA by the ASGPr, ^{125}I-ASOR was incubated with increasing concentrations of tcu-PA. Figure 8 shows that the binding of ^{125}I-ASOR could be competed for 90 % by tcu-PA with a K_i of 284 nM.

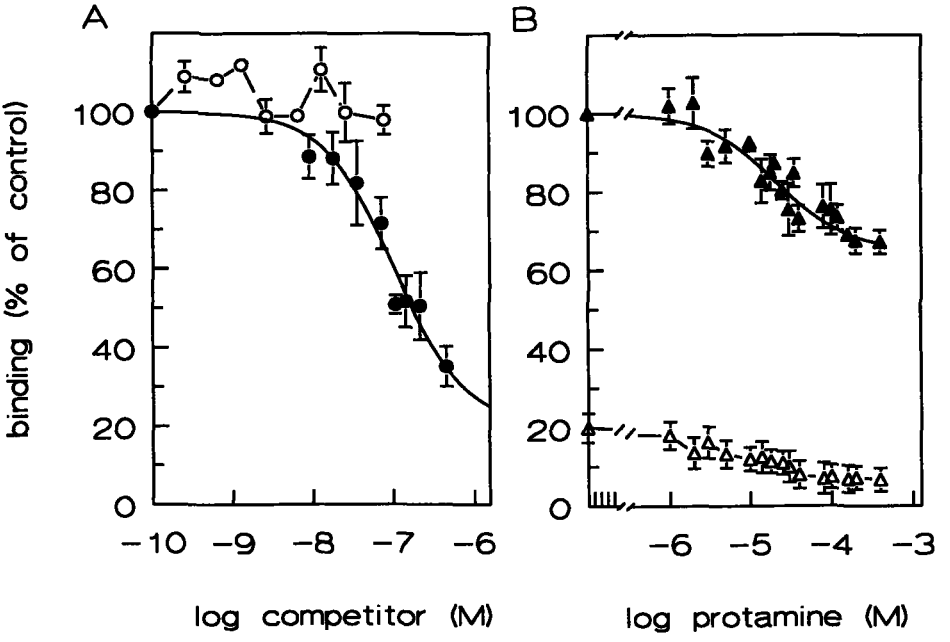

Figure 6. Effect of unlabelled tcu-PA, GST-RAP and protamine sulfate on the binding of ^{125}I-tcu-PA by rat parenchymal liver cells. Freshly isolated rat parenchymal liver cells were incubated for 2 h at 4 °C in the presence of 1 nM ^{125}I-tcu-PA with increasing amounts of unlabelled tcu-PA (●), GST-RAP (○, figure A) or protamine sulfate (▲, figure B). Furthermore, parenchymal liver cells were incubated with 1 nM ^{125}I-tcu-PA, 1000 nM ASOR and an increasing amount of protamine sulfate (Δ) (figure B). Data are expressed as % of control binding (10.2 ± 2.3 fmol/mg cell protein) and represent the mean (n = 3) ± S.D.

The recognition of tcu-PA by both ASGPr and HSPGs was further shown by incubation of parenchymal liver cells with ^{125}I-tcu-PA, 1000 nM ASOR and an increasing amount of protamine sulfate (figure 6B). It was determined that under the conditions that the interaction with the ASGPr was inhibited, protamine sulfate further reduced the cell association to 7 % of the control value.

Parenchymal liver cells were enzymatically treated with heparinase type I or chondroitin lyase ABC (chondroitinase), enzymes which digest heparan sulfate- and chondroitin sulfate proteoglycans, respectively. Heparinase treatment (abolishing the interaction with heparan sulfate proteoglycans) reduced the binding of tcu-PA to 73.4 ± 4.2 % of the control binding (very significant ($p<0.01$), (n = 3), data not shown). Treatment of the parenchymal liver cells with chondroitinase,

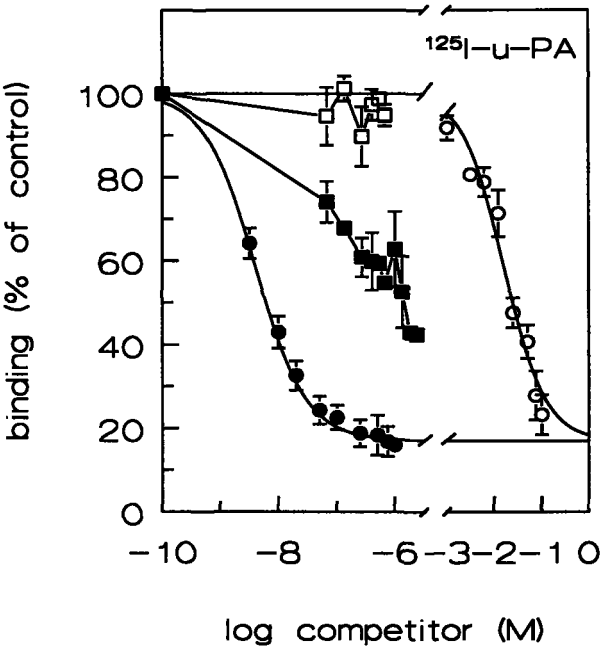

Figure 7. Effect of D-galactose, ASOR and anti-ASGP-receptor antibodies on the binding of ^{125}I-tcu-PA by rat parenchymal liver cells. Rat parenchymal liver cells were incubated for 2 h at 4 °C in the presence of 1 nM ^{125}I-tcu-PA and increasing amounts of unlabelled D-galactose (○), ASOR (●), rabbit IgG against rat asialoglycoprotein receptor (■) or non-immune rabbit IgG as control (□). Data are expressed as % of control binding (10.2 ± 2.3 fmol/mg cell protein) and represent the means of 2 (■, □) or 3 (○, ●) experiments ± S.D.

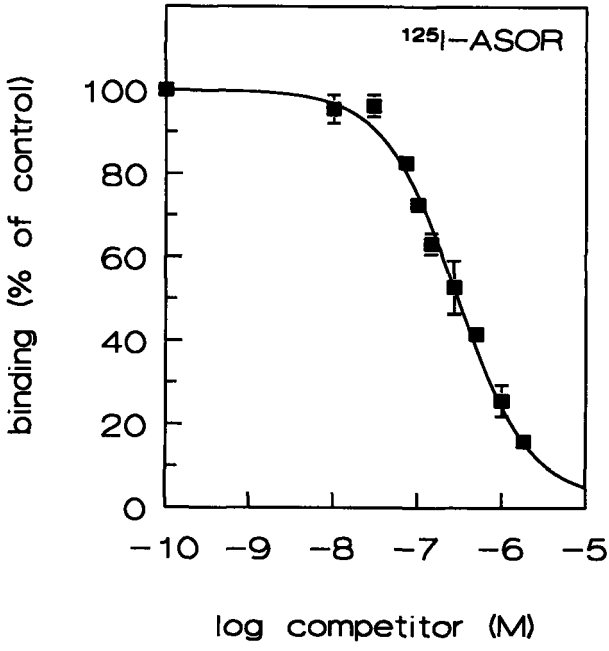

Figure 8. Effect of tcu-PA on the binding of ^{125}I-ASOR by rat parenchymal liver cells. Rat parenchymal liver cells were incubated for 2 h at 4 °C in the presence of 5 nM ^{125}I-ASOR and increasing amounts of unlabelled tcu-PA. Data are expressed as % of control binding (1.30 ± 0.02 pmol/mg cell protein) and represent the means of 2 experiments ± the variation of the individual experiments.

abolishing the interaction with chondroitin sulfate proteoglycans, resulted in a binding of 91.6 ± 4.7 % of the control binding (not significant, n = 3).

DISCUSSION

Recently, we reported that HMW-scu-PA isolated from a kidney cell culture is recognized by rat parenchymal liver cells via a lectin-like recognition site.[20] In the present study we show by isolation of a tcu-PA binding protein from rat and human liver using a tcu-PA-Sepharose column and subsequent immunoblotting, that urinary tcu-PA is recognized by the asialoglycoprotein receptor (ASGPr). The functional properties of the isolated human and rat receptor were similar to the properties of the ASGPr. The binding of ASGPr to immobilized tcu-PA was calcium dependent and acid-labile, as is shown before for the binding of various other ligands to the ASGPr.[46,51] Furthermore, ASGPr that bound to tcu-PA-Sepharose could be specifically eluted using D-galactose. The asialoglycoprotein receptor is found exclusively in parenchymal liver cells and only 10-25 % of the total number of ASGPr's is present on the cell surface.[52] The receptor is specific for terminal-clustered Gal or GalNAc residues, and it requires 2 or 3 sugars close together to give efficient binding with a preference for GalNAc residues.[52,53] Its physiological role is not clear but a major function of the ASGPr is the elimination of circulating glycoconjugates, which are rapidly endocytosed and degraded. Another suggested function of the ASGPr is the regulation of the intracellular routing of plasma glycoproteins from the Golgi (were they are synthesized) to the cell surface (were they are released in the circulation).[52,53]

The size and ratio of the proteins in the 40-60 kDa area corresponds well with the RHL-(rat hepatic lectin) 1-3 that are described by Drickamer *et al.*[53] (RHL-1 = 41.5 kDa, RHL-2 = 49 kDa, RHL-3 = 54 kDa). The RHL-1 protein was the predominant protein, while RHL-2 and RHL-3 were less abundant.[53] The intact ASGPr is a hexamer, but the stoichiometry of the different polypeptides in the oligomer is not clearly defined.[54,55] It is described that the RHL-1 and RHL-2/3 polypeptides are each associated into homo-oligodimers, resulting in a functional receptor protein that consists of a hetero-oligomeric complex of RHL-1 and RHL-2/3 multimers.[54,55] We also observed multimers of RHL-1, 2 and/or 3 in our experiments (figure 1, 2). These complexes turned to monomers when the protein samples were reduced by treatment with ß-mercaptoethanol.

In vitro studies showed that tcu-PA binds with a K_i of 103 nM to freshly isolated rat parenchymal liver cells. Binding could be inhibited for 80 % with D-galactose (K_i = 15.3 mM) or ASOR (K_i = 3.0 nM). The inhibition profile is characteristic of the ASGPr: mono-, bi-, tri- and tetra antennary galactose-terminal residues bind with increasing affinities, with dissociation constants of 10^{-3}, 10^{-6}, $5*10^{-9}$ and 10^{-9} M, respectively.[56] Further evidence for the involvement of the

ASGPr came from experiments in which the binding of ^{125}I-tcu-PA could be inhibited by increasing amounts of anti-RHL-antiserum.

Tcu-PA possesses a lower affinity for the ASGPr than ASOR (K_i = 3 nM for the competition of ^{125}I-tcu-PA by ASOR, K_i = 284 nM for the competition of ^{125}I-ASOR by tcu-PA). Recently Bergwerff *et al.*[57] showed that the isolated bi- and triantennary glycans of urinary tcu-PA contain predominantly GalNAcß1-4GlcNAcß in stead of Galß1-4GlcNAcß elements and that a portion of the antennae carry one or more sulfate groups. It was also shown that a large portion of the biantennary oligosaccharides terminate with one or two non-sulfated GalNAc residues. No triantennary oligosaccharides were described that carry terminal non-sulfated GalNAc residues. The observed affinity of tcu-PA for the ASGPr is in agreement with this. No significant difference in liver recognition was observed between bi- or triantennary oligosaccharides with terminal GalNAc residues.[58] Fiete *et al.*[59] identified a receptor present on rat hepatic Kupffer and endothelial cells that is responsible for the removal of the hormone lutropin, a glycoprotein that contains sulfated GalNAc residues. This receptor recognizes oligosaccharides terminating with (SO_4^-)-4GalNAcß1-4GlcNAcß1-2Manα. It is, however, unlikely that this receptor plays a major role in the uptake of tcu-PA since we have shown by cellular distribution experiments that the endothelial and Kupffer cells contribute only for 5 % to the liver uptake.

Compared to our previous observations[20], isolated parenchymal liver cells show a slightly lower affinity for urinary tcu-PA (= HMW-tcu-PA) than for HMW-scu-PA produced by cultured kidney cells (K_d = 40 nM). Despite the fact that they are derived from different sources and are single- and two-chain molecules, respectively, the recognition by the parenchymal liver cells is comparable. The 'lectin like' recognition as described before[20] is most likely to be mediated by the ASGPr.

The *in vivo* data show that tcu-PA is cleared rapidly from the plasma with a serum clearance of 1.6 ml/min. Urinary tcu-PA is mainly taken up by the liver (at least 40 % of the injected dose) in which the parenchymal cells are responsible for 94.5 % of the association. Besides the liver, the kidneys, skin and muscles contributed to the clearance of tcu-PA. Removal of the carbohydrate moiety of tcu-PA by incubation with N-glycosidase F or a preinjection of ASOR resulted in a 42 % reduction of the maximal liver uptake and a limited elongation of the plasma half-life. Since tcu-PA is still taken up by the liver when the interaction with the ASGPr is disturbed, another site then the ASGPr contributes to the liver association of urinary tcu-PA. Because of the heparin binding capacity of u-PA[48,49] the negatively charged proteoglycans present on the extracellular matrix of the parenchymal cells are likely candidates for this binding site. This was studied by inhibition of the parenchymal liver cell binding of tcu-PA to heparan sulfate proteoglycans (HSPGs) by the addition of an increasing concentration of protamine sulfate. It was found that protamine sulfate competed for 30 % of the parenchymal

liver cell binding of tcu-PA with low affinity (K_i = 21 μM). The interaction of tcu-PA with HSPGs was also shown by a 27 % reduction in parenchymal liver cell binding of ^{125}I-tcu-PA when the cells were pretreated with heparinase. The involvement of chondroitin sulfate proteoglycans in the binding of tcu-PA was not significant. Furthermore, the involvement of the ASGPr and HSPGs in the parenchymal liver cell binding of tcu-PA was demonstrated by the inhibition to 7 % of the control value when both receptors were inhibited at the same time. *In vivo* blockade of the interaction of HSPGs by preinjection of protamine sulfate, resulted in a small reduction in liver uptake (1.2-fold) but a significant delay in the serum clearance (1.8-fold) of tcu-PA. The delay in serum clearance is the result of the reduction in liver uptake plus a dramatic reduction in association of tcu-PA by the kidneys and skin. The inhibition of the binding of tcu-PA to both the ASGPr and the HSPGs by the combined preinjection of ASOR plus protamine sulfate resulted in a 58 % reduced liver uptake and 2-fold delay in serum decay. Candidates for the parenchymal liver cell recognition of tcu-PA by HSPGs are syndecan-1, 2, and 4.[60] The role of HSPGs in the turnover of tcu-PA may be a low affinity but high capacity, slowly internalizing recognition site. HSPGs however, may also facilitate transfer of u-PA initially bound to HSPGs to the rapidly internalizing ASGPr. Another role of HSPGs in the fibrinolysis may be the binding of u-PA followed by a slow release of u-PA in the circulation. In this way the maintenance of a constant concentration of (endogenous) u-PA in the blood is obtained.

Although the interaction of tcu-PA with the ASGPr and HSPGs is inhibited by ASOR and protamine sulfate, still 17 % of the injected ^{125}I-tcu-PA is associated to the liver (figure 4). Probably another recognition site is involved in this association. LRP, that is able to bind scu-PA and tcu-PA[18,20] did not contribute to the uptake of urinary tcu-PA by rat parenchymal liver cells, since GST-RAP was not able to compete for the binding of tcu-PA to the liver both *in vivo* and *in vitro*. This is in contrast to observations of Kounnas *et al.*[18] who showed that on HepG2 cells LRP mediates the uptake and degradation of HMW-scu-PA and HMW-tcu-PA. This difference can be explained by the different sources of the u-PA preparations that are used (Kounnas *et al.* used preparations obtained from Abbott, we used urinary tcu-PA from Serono). As we showed recently[20,47], the source of which u-PA obtained from is a determining factor in its receptor recognition. We showed that *E.coli* produced rscu-PA, that lacks the complex carbohydrate that possesses high affinity for the ASGPr, is recognized by LRP and not by the ASGPr on rat parenchymal liver cells. Henkin *et al.*[61] described clearance differences in rabbits for glycosylated u-PA preparations with different sialic acid contents. Glycosylated HMW-scu-PA produced by CHO cells, containing 2.5-3.0 molecules sialic acid per molecule was cleared more slowly than less sialyated HMW-scu-PA obtained from SP2/0 or human kidney cell cultures ($\leq$ 2.5 molecules sialic acid per molecule HMW-scu-PA). This indicates that the amount of sialic acid present on

the complex carbohydrate side chain of these u-PA preparations is responsible for differences in recognition (reflected in a different clearance).

No evidence for recognition of human tcu-PA by rat uPAR on Kupffer cells was present since the contribution of the Kupffer cells to the liver uptake of tcu-PA was only 2.4 %. In man, the Kupffer cell recognition of human tcu-PA may be more important as a result of species specificity. This species specificity has been described for the recognition of human u-PA by murine uPAR and reverse. It is based upon differences in the amino acid composition of the receptor binding sequence (residue 12-32) of u-PA.[62] Furthermore, it is also shown by Rabbani *et al.*[63] that the rat uPAR domain I, that is responsible for the interaction with u-PA, shows an amino acid homology of only 57 % with the corresponding human uPAR domain I sequence. Both differences in amino acid composition of the receptor binding sequence of u-PA and in the ligand recognition domain of uPAR may be responsible for the species specificity. Therefore in man, Kupffer cell recognition of human tcu-PA by uPAR may be more important.

In conclusion, this study shows that the parenchymal liver cells are primarily involved in the plasma clearance of urinary tcu-PA from the circulation. The identification of the receptor systems that are involved in the tcu-PA clearance such as the asialoglycoprotein receptor and heparan sulfate proteoglycans is essential in order to develop a strategy to prolong the half-life of tcu-PA during thrombolytic therapy.

ACKNOWLEDGEMENT

We would like to thank Dr. K. Drickamer for providing the antiserum against rat ASGP-receptor and Dr. J. Herz for providing the pGEX-RAP plasmid.
This study was supported by the Netherlands Heart Foundation (grant 92.139).

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Chapter 5

Characterization of the binding of urokinase-type plasminogen activator to the asialoglycoprotein receptor

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SUMMARY

In order to study the role of the asialoglycoprotein receptor (ASGPr) in the rapid plasma clearance of urokinase-type plasminogen activator (u-PA), a microtiter plate binding assay was developed using ASGPr purified from rat liver extracts. Urinary two-chain u-PA bound to immobilized ASGPr in a saturable manner with an EC_{50} of 0.2 μM. Binding was inhibited by rabbit antibodies against the ASGPr. In line with the known carbohydrate specificity of the ASGPr, GalNAc proved to be the most effective inhibitor from a series of monosaccharides, followed by Gal and Fuc, whereas GlcNAc was ineffective. Recent literature showed that the N-linked oligosaccharides of urinary tcu-PA do not terminate with the common Gal-GlcNAc element, but with a GalNAc-GlcNAc element which is partially sulfated. Sulfated forms of tcu-PA were separated from non-sulfated forms by using the lectin *Wisteria floribunda* agglutinin. Only the non-sulfated forms of u-PA (30 % of the total) appeared to bind to the ASGPr. From different u-PA preparations used for thrombolytic therapy only urinary u-PA and u-PA produced by kidney cell cultures strongly bound to the ASGPr, whereas (recombinant) u-PA expressed in mouse myeloma cells, Chinese hamster ovary cells or *E.coli* scarcely bound to the receptor. It is concluded that u-PA bearing non-sulfated GalNAc-GlcNAc elements is specifically recognized by the ASGPr present on liver cells.

INTRODUCTION

The balance between the blood coagulation system and the fibrinolytic system regulates haemostasis in the human body. Disregulation may lead either to bleeding or to thrombosis. The fibrinolytic system is composed of plasminogen, plasminogen activators and some proteinase inhibitors.[1] Plasminogen circulates at a relatively high concentration of 1.5 μM in blood. Plasminogen activators proteolytically convert plasminogen into plasmin, a serine proteinase which is responsible for the degradation of fibrin.[2] Two well described human plasminogen activators are tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA), which are homologous proteins and which circulate at relatively low concentrations of about 0.1 nM in blood.[3] The two activators have different mechanisms of action.[4]

During thrombolytic therapy the endogenous fibrinolytic system is stimulated by administration of plasminogen activators such as t-PA and u-PA.[5] Large amounts are administered during i.v. infusions, since both t-PA and u-PA have a short half-life in the circulation (5-10 min.) and are rapidly cleared predominantly by the liver.[6,7] The clearance mechanism may depend on the molecular form and the cellular source of the plasminogen activators.[8]

The native form of u-PA is a 55 kDa single-chain u-PA (scu-PA) which can be proteolytically cleaved between Lys_{158} and Ile_{159} into two-chain u-PA (tcu-PA).[3] Both scu-PA and tcu-PA consist of four different domains: the epidermal growth factor domain, the kringle domain, the connecting peptide domain and the proteinase domain. The growth factor domain is glycosylated with an O-linked fucose residue on Thr_{18}, while the proteinase domain is glycosylated with a N-linked carbohydrate chain on Asn_{302}.[9,10] Proteolytic elimination of the N-terminal part of the molecule (growth factor domain and kringle domain) results in low molecular weight u-PA of about 32 kDa. Current preparations of natural u-PA are isolated from urine or cell culture media. Recombinant forms of u-PA are produced by genetically transformed *E.coli* or genetically transformed eukaryotic cells, such as SP2/0 cells.

We are interested in the clearance mechanisms of the various forms of u-PA.[11] Recently we reported that non-glycosylated recombinant scu-PA expressed in *E.coli* is recognized by the Low Density Lipoprotein Receptor-related Protein (LRP) both *in vitro* on freshly isolated rat parenchymal liver cells[12] and *in vivo* in the rat.[13] In contrast, glycosylated u-PA from human embryonic kidney cells and human urine is recognized by rat parenchymal liver cells both *in vitro* and *in vivo* via a lectin-like recognition site which has been identified as the asialoglycoprotein receptor (ASGPr).[12,14] In the present study the binding of u-PA to the ASGPr is biochemically characterized using purified proteins. A preliminary report has been presented previously.[15]

MATERIALS AND METHODS

Materials

Urinary tcu-PA was obtained form Serono (Aubonne, Switzerland), Choay (Sanofi Winthrop, Maassluis, the Netherlands) or Kabi (Kabi Pharmacia, Woerden, the Netherlands). If the source is not specified, the Serono preparation (Ukidan) was used with a specific activity of 152 IU/μg protein. The protein concentration was determined at 280 nm, using an $E^{1\%}$ of 13.6.[16] Natural scu-PA, both the high molecular weight form and the low molecular weight form purified from human embryonic kidney (HEK) cell cultures, was kindly provided by Abbott Laboratories (Abbott Park, Ill).[17] Another natural scu-PA preparation was from a transformed human kidney cell line (TLC-598) and was kindly provided by Sandoz (Vienna, Austria).[18] Recombinant scu-PA preparations expressed in SP2/0 cells (both high and low molecular weight scu-PA), Chinese hamster ovary cells (low molecular weight scu-PA) and *E.coli* (Saruplase) were kindly donated respectively by Abbott Laboratories (Abbott Park, Ill), by Dr. H.R. Lijnen, University of Leuven (Leuven, Belgium)[19] and by Grühnenthal (Aachen, Germany). *Wisteria floribunda* agglutinin Sepharose was obtained from E.Y. Laboratories (San Mateo, Ca, USA). Rabbit antiserum against the rat asialoglycoprotein receptor was kindly donated by Dr. K. Drickamer (New York, USA). The IgG fractions of this antiserum and of non-immune rabbit serum were isolated using Protein A Sepharose chromatography (Pharmacia, Uppsala, Sweden). The monosaccharides N-acetyl-D-galactosamine (GalNAc), D-galactose (Gal), L-fucose (Fuc) and N-acetyl-D-glucosamine (GlcNAc) were bought from Sigma Chemical (St. Louis, Mo, USA).

Asialoglycoprotein receptor binding assay

The asialoglycoprotein receptor (ASGPr) was purified from rat liver extract using a urinary tcu-PA-Sepharose 4B column as described previously.[14] The only modification was that the ASGPr was eluted with a buffer (40 mM ammonium acetate, pH 6.0, containing 0.4 M NaCl and 0.1 mM phenylmethylsulfonyl fluoride) without Triton X-100, which allowed immobilization of the ASGPr onto the wells of polyvinylchloride microtiter plates. Immobilization of ASGPr was achieved by an overnight incubation at 4 °C of 50 ng ASGPr in 100 μl 50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl and 5 mM $CaCl_2$ (coating buffer) per well. Blank wells were treated similarly but without ASGPr. The immobilized ASGPr and its blank were washed with coating buffer supplemented with 1 mg/ml BSA and 0.5 % (v/v) Tween 80 (binding buffer) for 0.5 h at room temperature and then incubated with 100 μl u-PA in binding buffer for 2 h at room temperature. Bound u-PA was washed three times with binding buffer and once with binding buffer without NaCl and then quantitated by measuring the plasminogen activator activity. To this end 100 μl of a mixture of Glu-plasminogen (0.5 μM final concentration) and the plasmin substrate H-D-Val-Leu-Lys-p-nitroanilide (0.6 mM final concentration) in 50 mM Tris-HCl, pH 7.4, containing 5 mM EDTA, 1 mg/ml BSA and 0.5 % (v/v) Tween 80 was added to the wells (EDTA dissociates bound tcu-PA) and the microtiter plates were incubated at 25 °C. The absorbance at 405 nm was repeatedly measured by using a microtiter plate reader. The amounts of bound u-PA (expressed as IU or ng per ml) were determined by comparing the absorbance at 405 nm with the absorbance obtained with serial dilutions of u-PA incubated with plasminogen and the plasmin substrate in parallel wells. Specific binding was obtained by subtracting u-PA bound to blank wells from u-PA bound to ASGPr-coated wells. The binding experiments were performed at least in duplicate. Binding curves were analyzed as sigmoidal curves by using non-linear regression analysis (SlideWrite Plus Program, Advance Graphics Software, Carlsbad, Ca), yielding data for half-maximal binding (EC_{50}) and maximal binding (Bmax).

***Wisteria floribunda* agglutinin (WFA) chromatography**

Molecular forms of tcu-PA with free terminating GalNAc residues were separated from forms with substituted (predominantly sulfated) GalNAc residues by means of a WFA-Sepharose 4B column. A column containing 2 ml of gel was washed four times alternately with high and low pH buffer solutions to remove loosely bound WFA and then equilibrated with 50 mM Hepes buffer, pH 7.5, containing 0.5 M NaCl and 0.01 % (v/v) Tween 80. An aliquot of 1.3 mg urinary tcu-PA (Ukidan) was applied onto the column at 4°C. After washing unbound tcu-PA from the column with equilibration buffer, bound tcu-PA was eluted with 50 mM lactose in the same buffer.

RESULTS

Asialoglycoprotein receptor binding assay

Microtiter plates were coated with varying amounts of an asialoglycoprotein receptor (ASGPr) preparation purified from rat liver, and incubated with 50 IU/ml urinary tcu-PA. Bound tcu-PA was quantitated and a suitable amount of ASGPr (50 ng) was selected and used throughout the study. Figure 1 shows tcu-PA binding to the immobilized receptor at increasing tcu-PA concentrations. After subtraction of the nonspecific binding obtained from wells without ASGPr, a saturable binding was observed with an EC_{50} value of 1650 IU/ml, which corresponds to 10.9 μg/ml or 0.2 μM. In figure 2 it is shown that the specific binding of tcu-PA could be completely inhibited with the IgG fraction of an antiserum against rat ASGPr, whereas control antibodies had no significant effect.

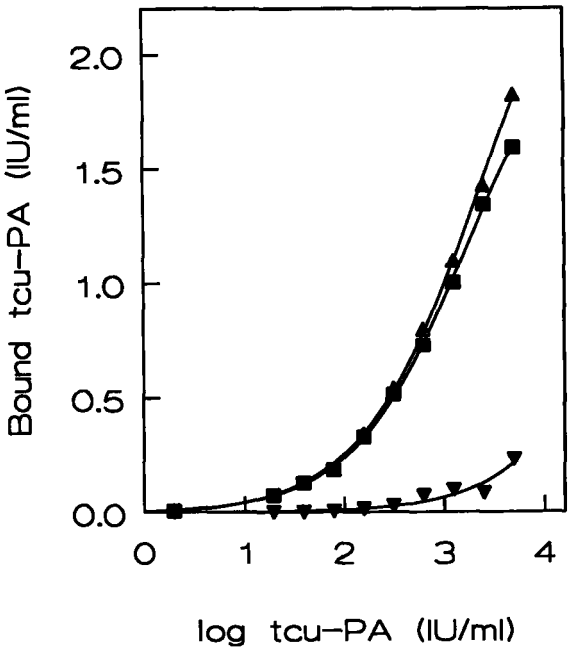

Figure 1. Binding of urinary tcu-PA to the asialoglycoprotein receptor. Increasing concentrations of tcu-PA (Ukidan from Serono) were incubated in microtiter plate wells coated with isolated asialoglycoprotein receptor (ASGPr) and tcu-PA binding was determined as described in "Materials and Methods". Specific tcu-PA binding (■) was calculated by correcting total tcu-PA binding (▲) with non-specific binding (▼) measured in wells without ASGPr.

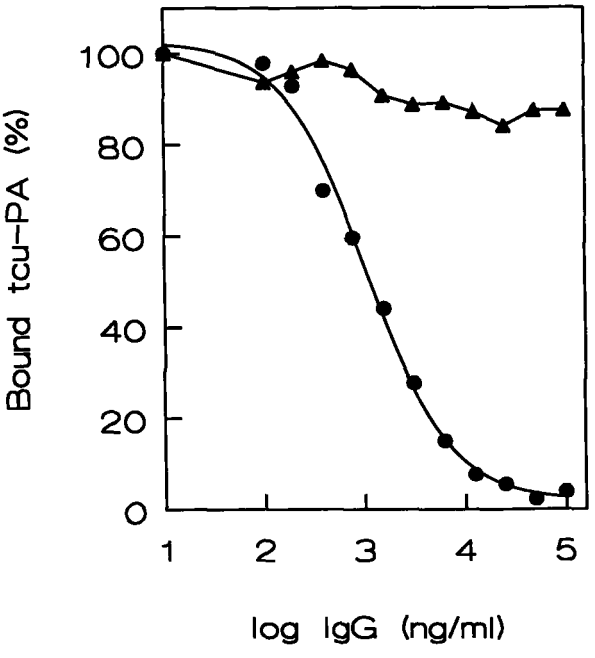

Figure 2. Inhibition of tcu-PA binding to the ASGPr by anti-ASGPr antibodies. Urinary tcu-PA (50 IU/ml) was incubated with the immobilized ASGPr in the presence of increasing amounts of rabbit IgG against the ASGPr (●) or rabbit non-immune IgG (▲). Specific tcu-PA binding was determined and expressed as % of tcu-PA binding in the absence of IgG. The anti-ASGPr IgG inhibition curve was analyzed as a sigmoidal curve by using nonlinear regression analysis.

Finally, the specificity of tcu-PA binding to the immobilized ASGPr was investigated by measuring the inhibition by a series of monosaccharides. Figure 3 shows that GalNAc had a strong inhibitory effect (IC_{50} = 37 μM), Gal a moderate effect (IC_{50} = 2.3 mM), Fuc a weak effect (IC_{50} > 10 mM), while GLcNAc had no effect at all on the binding of tcu-PA to the immobilized ASGPr (up to a concentration of 10 mM) on the binding of tcu-PA to the immobilized ASGPr.

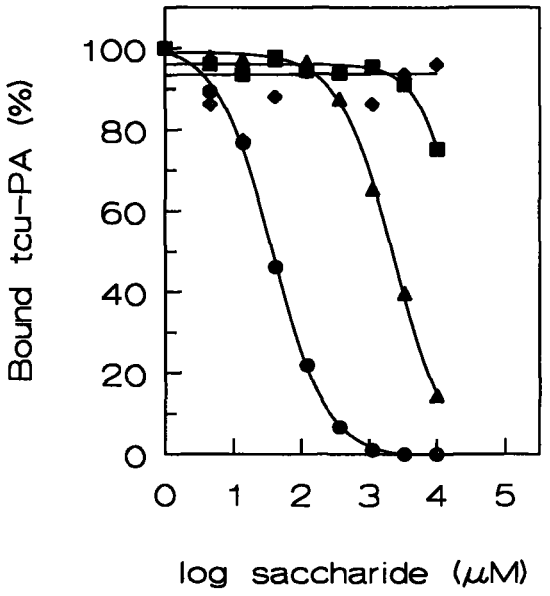

Figure 3. Inhibition of tcu-PA binding to the ASGPr by monosaccharides. Urinary tcu-PA (50 IU/ml) was incubated with the immobilized ASGPr in the presence of increasing concentrations of the monosaccharides GalNAc (●), Gal (▲), Fuc (■) or GlcNAc (◆). Specific tcu-PA binding was determined and expressed as % of tcu-PA binding in the absence of monosaccharides. Inhibition curves were analyzed as sigmoidal curves by using nonlinear regression analysis.

Carbohydrate heterogeneity of urinary tcu-PA

A recent study of Bergwerff *et al.*[20] on the primary structure of the N-linked carbohydrate chain of urinary tcu-PA from the same source as used here (Serono) showed the presence of predominantly terminal GalNAcβ1-4GlcNAcβ elements which are partially sulfated. To separate molecular forms of tcu-PA with free terminal GalNAc residues from forms with sulfated (or sialylated) terminal GalNAc residues, urinary tcu-PA was chromatographed on *Wisteria floribunda* agglutinin (WFA)-Sepharose, which binds oligosaccharides with free terminal β1,4-linked GalNAc moieties.[21,22] Figure 4 shows that about 70 % of the tcu-PA protein passed through the column (sulfated forms), whereas about 30 % bound to the WFA-Sepharose and was eluted with lactose (non-sulfated forms). The plasminogen activator activity per mg of protein was similar for the two fractions The activity of the non-sulfated tcu-PA fraction was 69 ± 4 % of the activity of the

sulfated tcu-PA fraction (n = 4; not shown). Binding of the two fractions to the immobilized ASGPr is shown in figure 5. The EC_{50} of the non-sulfated tcu-PA fraction was 2.2-fold lower than that of the original mixture (4.0 versus 8.7 μg/ml) and about 100-fold lower than that of the sulfated tcu-PA fraction. The weak binding of the sulfated tcu-PA fraction may be ascribed either to a low affinity of sulfated tcu-PA for the ASGPr, or to a contamination of the preparation with about 1 % non-sulfated tcu-PA. In the latter case sulfated tcu-PA has no measurable affinity for the ASGPr.

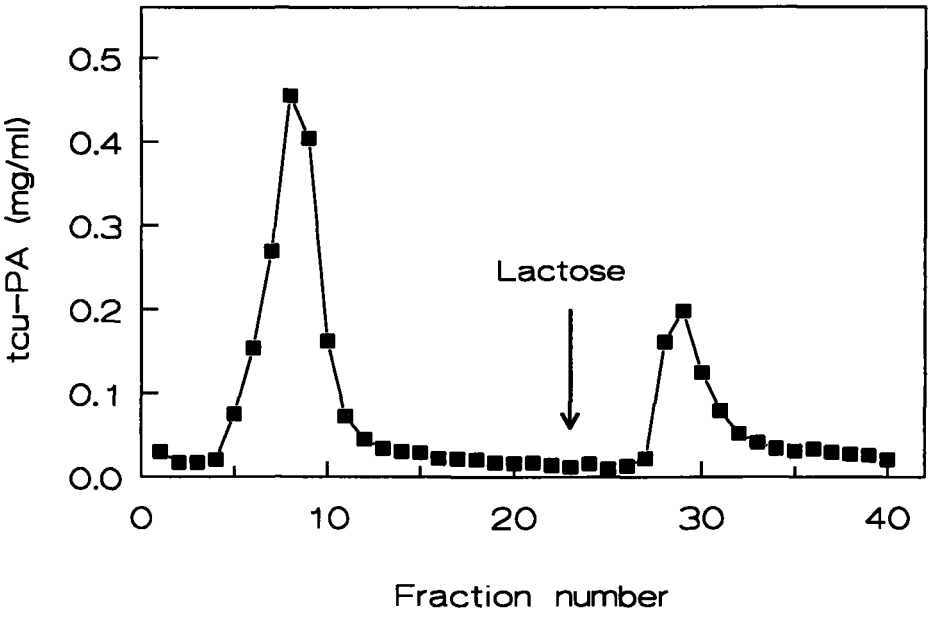

Figure 4. Chromatography of tcu-PA on WFA-Sepharose 4B. An aliquot of 1.3 mg urinary tcu-PA was chromatographed as described under "Materials and Methods". The tcu-PA concentrations in the fractions were determined at 280 nm. Fractions 5-11 and fractions 28-31 were pooled and used as sulfated and non-sulfated tcu-PA, respectively.

ASGPr binding of u-PA preparations from different sources

Because the structure of the carbohydrate moiety of u-PA might strongly depend on the cellular source, various u-PA preparations were tested in the ASGPr binding assay. Figure 6 shows the binding results, expressed as maximal binding divided by the EC_{50}. Two other commercial tcu-PA preparations obtained from urine (Choay and Kabi) bound as well as tcu-PA from Serono to the ASGPr.

Natural scu-PA preparations from a human embryonic kidney (HEK) cell culture and from a kidney tumor cell line (TLC-598) showed a similar affinity to the ASGPr as the three urinary tcu-PA preparations. In contrast, recombinant scu-PA obtained from the mouse myeloma cell line SP2/0 revealed reduced binding (due to a 5-10 fold increased EC_{50} value; not shown). Recombinant scu-PA expressed in Chinese hamster ovary (CHO) cells or in *E.coli* did not bind to the ASGPr. Low molecular weight scu-PA from HEK cells or from SP2/0 cells bound to the ASGPr to a similar extent as their high molecular weight counterparts, indicating that the growth factor and kringle domains of u-PA are not significantly involved in the binding to the ASGPr (figure 6).

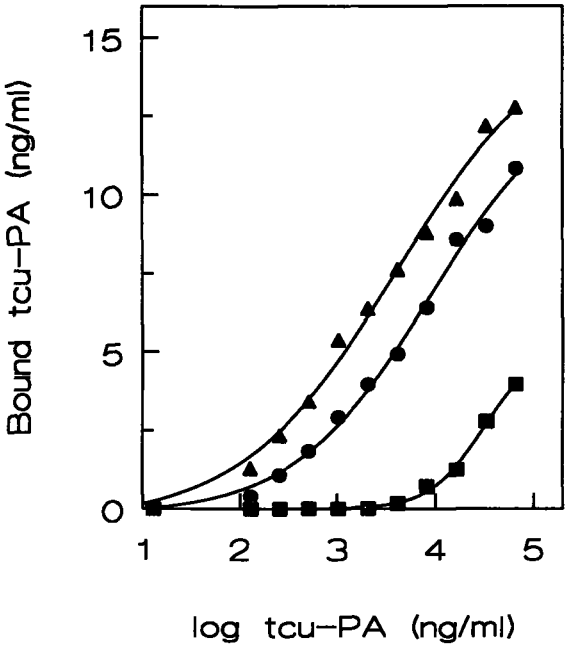

Figure 5. Effect of sulfation of tcu-PA on the interaction with the ASGPr. Increasing concentrations of urinary tcu-PA (●), its sulfated fraction (■) and its non-sulfated fraction (▲) were incubated with immobilized ASGPr and specific binding was determined as described in figure 1.

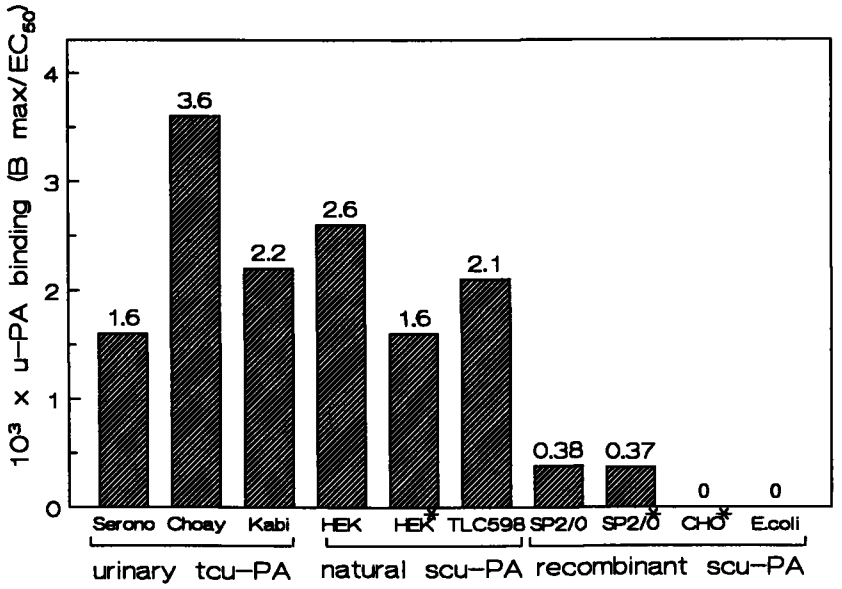

Figure 6. Binding of various u-PA preparations to the ASGPr. Specific binding of each u-PA preparation was measured as described in figure 1 and expressed as the ratio between maximal binding (Bmax) and EC_{50}. The asterisks at HEK, SP2/0 and CHO indicate that the binding of low molecular scu-PA was determined.

DISCUSSION

This study provides biochemical evidence that u-PA is specifically recognized by the ASGPr and that non-sulfated terminal GalNAc residues in the carbohydrate part of u-PA are essential for the high affinity interaction.

The ASGPr or Gal/GalNAc-specific lectin of mammalian liver is one of the best-studied receptors involved in receptor-mediated endocytosis (reviewed in reference 23 and 24). The receptor mediates clearance of serum glycoproteins from which the terminal sialic acid residues have been removed resulting in exposed terminal galactose residues. As GalNAc has a higher affinity to the ASGPr than Gal, neoglycopeptides containing terminal GalNAc residues are high affinity ligands[25] which can be used for *in vivo* targeting to the ASGPr.[26]

An important step in the present study was the development of a microtiter plate assay for ASGPr binding studies. Detergent-free ASGPr from rat liver was immobilized onto the microtiter plates and then incubated with urinary tcu-PA,

which bound to the receptor with an EC_{50}-value of 0.2 μM. The specificity of the binding of tcu-PA to the ASGPr was demonstrated with specific antibodies against the receptor which inhibited the binding completely. Furthermore, a series of monosaccharides inhibited the binding in a manner that is typical for the ASGPr: strong inhibition by GalNAc and Gal, weak inhibition by Fuc and no significant inhibition by GlcNAc.[27]

Urinary tcu-PA used in this study was Ukidan from Serono. The primary structure of the N-linked complex-type carbohydrate of this material was recently determined in detail.[20,28] The glycans contain predominantly GalNAcβ1-4GlcNAcβ instead of the common Galβ1-4GlcNAcβ elements. A large portion of the GalNAc residues is sulfated at O4, a small portion bears sialic acid at O6 and a third portion is not substituted. Most of the glycans are biantennary and carry either no or two sulfate groups (less than 2 % of the u-PA mixture carries a biantenna terminating with one GalNAc and one sulfated GalNAc). Because sulfation, as sialylation, might prevent the interaction with the ASGPr, sulfated forms of tcu-PA were separated from non-sulfated forms by means of chromatography on WFA-Sepharose 4B, which specifically binds free terminating GalNAc residues.[21,22] Indeed, it was demonstrated that only the non-sulfated fraction of urinary tcu-PA is recognized by the ASGPr.

Next questions were how widely this GalNAcβ1-4GlcNAcβ element is distributed in various u-PA preparations and how strongly other u-PA preparations bind to the ASGPr. It was established that other commercially available urinary tcu-PA preparations (from Choay and Kabi) bound to the ASGPr as well as Ukidan. Furthermore, it was determined that scu-PA preparations obtained from different kidney cell cultures also bound well to the ASGPr. Taken together, these results indicate that kidney cells apparently possess the GalNAc-transferase activity required for the incorporation of GalNAc into the N-glycans of u-PA. The identity of this GalNAc-transferase is still unclear.[20,29] Recombinant scu-PA from the mouse myeloma cell line SP2/0 and from CHO cells showed a poor binding to the ASGPr and thus exposes no or only small amounts of terminal GalNAc residues. Indeed, the antennary element GalNAcß1-4GlcNAcß does not occur in a large series of recombinant glycoproteins produced in CHO cells[30]. In aditition, recombinant scu-PA preparations from CHO cells have been found to contain high amounts of sialic acid.[31] As expected, recombinant scu-PA expressed in *E.coli*, did not bind to the ASGPr, because of the absence of glycosylation.

Sulfation of terminal GalNAc residues may turn a relatively common structural motif into a unique carbohydrate with specific properties (reviewed in reference 32). One example is the control of the circulatory half-life of luteinizing hormone (LH), as the sulfated form of this hormone is recognized by a receptor specific for terminal GalNAc-4-SO_4 on liver endothelial and Kupffer cells.[33] Because of this recognition, sulfated LH has a shorter half-life than recombinant LH produced by CHO cells. Desulfation of LH leads to a further decrease of the half-

life.[34] Sulfation of the oligosaccharides of u-PA also affects the circulatory half-life. It prevents the interaction of urinary tcu-PA with the ASGPr, as demonstrated in the present and an accompanying study[35], which leads to a prolonged circulatory half-life *in vivo*. Sulfated tcu-PA is not significantly recognized by the receptor for luteinizing hormone.

In conclusion, this study shows that sulfation of the terminal GalNAc residues of urinary tcu-PA determines the recognition by the liver asialoglycoprotein receptor. Non-sulfated GalNAc-terminal tcu-PA is recognized by the asialoglycoprotein receptor, while sulfated GalNac-terminal tcu-PA is not recognized by the asialoglycoprotein receptor.

ACKNOWLEDGEMENT

This work was supported by Grant 92.139 from the Netherlands Heart Foundation.

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Chapter 6

Sulfation of the oligosaccharides of urinary urokinase determines the receptor recognition on rat liver cells

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SUMMARY

Recently we reported that glycosylated urinary urokinase (tcu-PA) is recognized by the asialoglycoprotein receptor (ASGPr) and heparan sulfate proteoglycans (HSPGs) on rat parenchymal liver cells. The carbohydrate moiety of urinary tcu-PA is a mixture of sulfated and non-sulfated GalNAcß1-4GlcNAcß1-2Man terminal bi- and triantennary glycans and we determined whether sulfation determines the interaction with the cellular binding sites. The tcu-PA preparation was therefore separated by means of a Wisteria floribunda agglutinin Sepharose column to obtain a sulfated tcu-PA (70 % of the preparation) and a non-sulfated tcu-PA fraction (30 % of the preparation).

In vitro studies demonstrated that non-sulfated tcu-PA bound with a K_i of 26.7 ± 0.6 nM to parenchymal liver cells. Since the binding of non-sulfated tcu-PA could be efficiently inhibited by asialo-orosomucoid (ASOR) (K_i = 10.0 ± 1.1 nM) and the binding of ^{125}I-ASOR could be displaced by non-sulfated tcu-PA, we conclude that non-sulfated tcu-PA binds to the ASGPr. Sulfated tcu-PA showed a 20-fold reduced parenchymal cell binding (3.3 ± 0.3 fmol/mg cell protein and 65.4 ± 5.0 fmol/mg cell protein for sulfated and non-sulfated tcu-PA, respectively).

Sulfated ^{125}I-tcu-PA was rapidly cleared from the circulation in rats (clearance 1.8 ± 0.1 ml/min) with a maximal liver uptake of 33.7 ± 4.1 % of the injected dose (% i.d.). Non-sulfated ^{125}I-tcu-PA was cleared 1.4-fold more rapidly (clearance 2.5 ± 0.1 ml/min) and the maximal liver uptake was 67.6 ± 0.3 % i.d. Both tcu-PA preparations were almost exclusively taken up by the parenchymal liver cells. A preinjection of ASOR resulted in a 70 % reduction in the maximal liver uptake and a 1.9-fold delayed serum clearance of non-sulfated tcu-PA. A preinjection of protamine sulfate, an inhibitor of HSPGs, reduced the maximal liver uptake of sulfated tcu-PA 1.8-fold and reduced the clearance 1.6-fold. Removal of the sulfated carbohydrate moiety did not influence the HSPGs interaction, which indicates that the sulfated carbohydrate moiety is not involved in the recognition of tcu-PA by the HSPGs. Non-sulfated tcu-PA appears not to be recognized by HSPGs unless the carbohydrate moiety was removed by N-glycosidase F treatment. This indicates that removal of it's high affinity preference receptor site (GalNAc terminal glycan) is needed before tcu-PA can be recognized via its net positively charged protein core by HSPGs.

It is concluded that sulfation of the GalNAc terminal bi- and triantennary glycans of urinary tcu-PA is decisive for the recognition by parenchymal liver cells. Non-sulfated GalNAc-tcu-PA is recognized by the asialoglycoprotein receptor, while sulfation of the GalNAc antennae prevents the interaction with the asialoglycoprotein receptor and results primarily in the interaction with heparan sulfate proteoglycans.

INTRODUCTION

Urokinase-type plasminogen activator (u-PA) is a highly specific serine protease that converts the pro-enzyme plasminogen into the active plasmin. This enzyme plays an important role in the degradation of fibrin clots. Because of its capacity to degrade fibrin via plasminogen activation, u-PA is an attractive thrombolytic agent. The u-PA preparations that are used in the clinic are currently extracted from urine.[1,2] A major drawback in the use of u-PA as thrombolytic agent is its rapid clearance from the circulation.[3,4] The liver and to a lesser extent the kidneys have been identified to play a major role in the uptake of high molecular weight- (HMW) and low molecular weight (LMW) u-PA in monkeys, rabbits and rats.[3-7]

Urokinase is produced as a single chain protein (scu-PA)[8] with a molecular mass of 55,000 Da (HMW-scu-PA) by e.g. cultured kidney cells[9,10], tumor cells[11] and fibroblasts.[12] Proteolytic cleavage of scu-PA between Lys_{158} and Ile_{159} by plasmin or kallikrein results in the conversion into an active two-chain form (tcu-PA). When expressed in eukaryotic cells, u-PA is secreted as a glycoprotein with an O-linked α-fucose at Thr_{18}[13,14] and a complex N-linked oligosaccharide at Asn_{302}.[15] Bergwerff *et al.*[16] showed that the isolated bi- and triantennary glycans of urinary tcu-PA contain GalNAcß1-4GlcNAcß1-2Man at the terminus of their antennae. A large portion of the GalNAc antennae carries SO_4 at the C4 position, or to a lesser extent bears N-acetylneuraminic acid at the C6 position. The GlcNAc residue can be fucosylated at the C3 position. It was also shown that a large portion of the biantennary oligosaccharides terminate with two non-sulfated GalNAc residues. A small portion (less than 2 % of the tcu-PA mixture[16]) of the biantennary oligosaccharides terminate with one GalNAc and one SO_4-GalNAc. Triantennary oligosaccharides with terminal free GalNAc residues were not found. Biantennary oligosaccharides with terminal non-sulfated GalNAc residues as being present in urinary tcu-PA have been shown to be good ligands for the asialoglycoprotein receptor (ASGPr).[17] Recently we described that this urinary tcu-PA is recognized by the ASGPr and heparan sulfate proteoglycans (HSPGs) on the parenchymal liver cells.[18]

In the present study, we further investigated the role of sulfation of glycosylated urinary tcu-PA by separating tcu-PA into two fractions that contain sulfated and non-sulfated GalNAc bearing glycans, respectively. It is shown that sulfated tcu-PA (70 % of the preparation) is recognized by HSPGs while non-sulfated tcu-PA, with GalNAc terminal glycans (30 % of the preparation), is exclusively recognized by the ASGPr on the parenchymal liver cells. It is suggested that sulfation of the GalNAc terminal glycans of urinary tcu-PA prevents tcu-PA binding to the ASGPr and allows recognition by HSPGs.

MATERIALS AND METHODS

Materials

Two-chain urinary urokinase (= HMW-tcu-PA, "Ukidan") was from Serono (Aubonne, Switzerland). Bovine serum albumin (BSA fraction V), collagenase type I and IV, human orosomucoid, neuraminidase (agarose bound) and protamine sulfate were from Sigma Chemical Co., (St. Louis, MO, USA). Dulbecco's modified Eagle medium (DMEM) was from Gibco (Irvine, Scotland). ^{125}I (carrier free) in NaOH was obtained from Amersham (Buckinghamshire, United Kingdom). Pronase and N-glycosidase F were bought from Boehringer Mannheim (Mannheim, Germany). Glu-Gly-Arg Chloromethyl Ketone dihydrochloride (GGACK) was obtained from Calbiochem (San Diego, USA). Wisteria floribunda agglutinin Sepharose was obtained from E.Y. Laboratories (San Maleo, USA). All other chemicals were of analytical grade.

Separation of sulfated tcu-PA and non-sulfated tcu-PA by WFA-Sepharose

Separation of sulfated tcu-PA and non-sulfated tcu-PA was performed as described by Rijken *et al.*[19] In brief, urinary tcu-PA was loaded to a Wisteria floribunda agglutinin Sepharose column (WFA-Sepharose) and washed with 50 mM HEPES, pH 7.5, containing 0.5 M NaCl and 0.01 % (v/v) Tween 80. The unbound fraction (70 % of the preparation) consisted of glycosylated tcu-PA that bears terminal SO_4-GalNAcs (sulfated tcu-PA). Non-sulfated GalNAc-tcu-PA was eluted from the WFA-Sepharose column by using the same buffer containing 50 mM lactose. The eluate (30 % of the preparation) consisted of glycosylated tcu-PA with free terminal GalNAc on its antennae (non-sulfated tcu-PA).

Protein iodination

tcu-PA was iodinated as described earlier[20] using the iodogen method, resulting in a specific radioactivity of 2200 cpm/ng (sulfated tcu-PA) and 1800 cpm/ng (non-sulfated tcu-PA). Asialo-orosomucoid (ASOR), that was prepared by treating orosomucoid with neuraminidase-agarose, was iodinated[20] resulting in a specific radioactivity of 640 cpm/ng.

Treatment of tcu-PA with N-glycosidase F

Radiolabelled or non-radiolabelled sulfated tcu-PA or non-sulfated tcu-PA (100 μg/ml) was treated for 18 h at 37 °C with 4 U/ml N-glycosidase F in 50 mM phosphate buffer, 10 mM EDTA, 10 mM NaN_3, pH 7.2, followed by dialysis against PBS as described before[20]. Removal of the carbohydrate moiety was checked on 10 % SDS-PAGE.

In vitro binding of tcu-PA

9-10 weeks old male Wistar rats (180-210 g, Broekman Institute BV, Someren, The Netherlands) were anaesthetized by intraperitoneal injection of 15-20 mg of sodium pentobarbital and parenchymal liver cells were isolated by perfusion of the liver for 9 min with collagenase (type IV, 0.05% (w/v)) at 37 °C by the method of Seglen[21], modified as described previously.[22] The obtained parenchymal liver cells were ≥ 95% viable as judged by 0.2 % trypan blue exclusion and ≥ 99 % pure as judged by light microscopy. For binding studies the parenchymal liver cells were resuspended in DMEM supplemented with 2 % BSA (pH 7.4).

Parenchymal cells (1-2 mg cell protein/ml) were incubated with 1 nM sulfated ^{125}I-tcu-PA or 1 nM non-sulfated ^{125}I-tcu-PA with or without competitors at the indicated concentrations in a volume of 0.5 ml DMEM containing 2 % (w/v) BSA pH 7.4. Incubations were carried out in plastic containers (Kartell, Milan, Italy) for 2 h at 4 °C and continuously shaken (150 rpm, Adolf Kuhner, Basel, Switzerland). At the end of the incubations, cells were washed twice at 4 °C and centrifuged for 30 sec at 50 xg with buffer containing 10 mM Tris-HCl, 0.15 M NaCl, 5 mM $CaCl_2$, 0.2 % BSA, pH 7.4 (at 4 °C), followed by one wash with the same buffer without BSA. Finally, cells were resuspended in this buffer (without BSA) and cell bound radioactivity was counted in a gamma counter. The amount of cell protein was determined as described by Lowry *et al.*[23] with BSA as standard.

Serum decay and tissue uptake

9-10 weeks old male Wistar rats (180-210 g, Broekman Institute BV, Someren, The Netherlands) were anaesthetized by intraperitoneal injection of 15-20 mg of sodium pentobarbital. The abdomen was opened, sulfated or non-sulfated ^{125}I-tcu-PA (1.0 μg/kg body weight) that was inactivated by incubation for 10 min at room temperature with 2 μM of GGACK to prevent complex formation with plasminogen activator inhibitor-type 1 (PAI-1) was injected via the inferior vena cava and at the indicated times liver lobules were tied off and 0.3 ml blood samples were taken from the inferior vena cava and allowed to clot for 30 min. The body temperature of the rats was kept at 37 °C using an infrared lamp. The respiration was facilitated by administration of carbogen 95/5 (95 % O_2, 5 % CO_2). The blood samples were centrifuged for 2 min at 10,000 xg and 100 μl serum samples were counted for radioactivity. The excised liver lobules (which totally weighed less than 15 % of the liver mass) were weighed and the radioactivity was counted. Total liver uptake was determined by taking into account, the measured liver weight at the end of the experiment. The total amount of radioactivity in the serum and liver lobules was determined as described previously.[24-26]

Uptake of ^{125}I-tcu-PA in other organs was determined by removal and weighing the organs at 10 min after injection. Radioactivity was counted and corrected for the radioactivity in the serum. Values for the serum content of tissues were calculated by determining the amounts of radioactivity present in tissues after injection of radiolabelled serum albumin.[24]

Liver cell distribution

Rats were anaesthetized and injected with radiolabelled ligands as described above. After 10 min circulation, the liver was perfused at 8 °C with collagenase. Parenchymal (PC), endothelial (EC) and Kupffer cells (KC) were isolated by centrifugation, followed by counterflow centrifugal elutriation at 4 °C as described in detail elsewhere.[27-29] The contributions of the different liver cell types to the total hepatic uptake of the injected ligand were calculated with the assumption that parenchymal, endothelial and Kupffer cells account for 92.5, 3.3 and 2.5% of the total liver protein mass, respectively.[27-29] As found for a number of substrates[27-29], 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.

Pharmacokinetic analysis and statistics

Plasma clearance curves were analyzed by computerized nonlinear fitting following a biphasic clearance model ($t_{½\alpha}$, $t_{½\beta}$, AUC_{0-29}) using the program Graphpad Prism (Graphpad Software Inc., San Diego, U.S.A.). The clearance was calculated by the formula clearance = dose/AUC_{0-29}. Results are given as mean ± the variation in the experiments. Binding parameters (K_i) were determined from displacement curves using a sigmoid model of the binding data with a "Hill slope" of 1.0 representing a one site binding model using the same program.

RESULTS

Serum decay and liver uptake of sulfated tcu-PA and non-sulfated tcu-PA

Sulfated or non-sulfated tcu-PA separated by a WFA-Sepharose column were injected into rats (1.0 μg/kg body weight) and their fate was studied. As shown in figure 1 non-sulfated ^{125}I-tcu-PA is more rapidly cleared from the circulation than sulfated ^{125}I-tcu-PA. A clearance of 2.5 ± 0.1 ml/min (non-sulfated tcu-PA) and 1.8 ± 0.1 ml/min (sulfated tcu-PA) was calculated. The faster clearance of non-sulfated

^{125}I-tcu-PA was accompanied by a higher uptake in the liver. The maximal liver uptake was observed at 10 min after injection of 67.6 ± 0.3 % of the injected dose (% i.d.) after which the liver-associated radioactivity decreased to 33.5 ± 0.2 % i.d. at 30 min, due to degradation of tcu-PA. The liver uptake of sulfated ^{125}I-tcu-PA reached a maximum of 33.7 ± 4.1 % i.d. also at 10 min after injection, after which the liver-associated radioactivity slowly decreased to 20.2 ± 2.9 % i.d. at 30 min after injection. Urinary tcu-PA not separated in non-sulfated and sulfated fractions by WFA-Sepharose shows an intermediate liver uptake (40 % i.d. at 10 min after injection) and a clearance of 1.6 ml/min.[18]

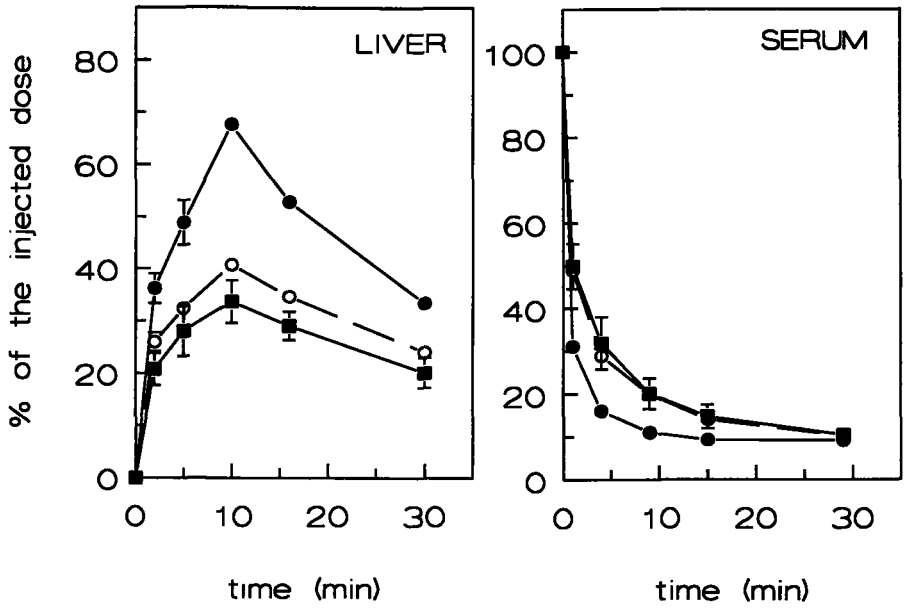

Figure 1. Liver uptake and serum decay of sulfated ^{125}I-tcu-PA and non-sulfated ^{125}I-tcu-PA. Rats were injected intravenously with 1.0 μg/kg body weight sulfated ^{125}I-tcu-PA (■) or non-sulfated ^{125}I-tcu-PA (●). At the indicated times, radioactivity in the liver and serum were determined. The open circles (○) represent the liver uptake and serum clearance of the original mixture of sulfated and non-sulfated ^{125}I-tcu-PA as has been described previously.[15] Data are expressed as % of the injected dose are mean (n = 2) ± the variation of the individual experiments. When the variation is not visible, it fells within the symbol size.

Liver cell distribution of sulfated-tcu-PA and non-sulfated-tcu-PA

In order to analyze the contribution of the different liver cell types in the recognition of sulfated ^{125}I-tcu-PA and non-sulfated ^{125}I-tcu-PA, we isolated parenchymal, endothelial and Kupffer cells at 10 min after injection of sulfated ^{125}I-tcu-PA and non-sulfated ^{125}I-tcu-PA (1.0 μg/kg body weight). Table I shows that the parenchymal cells were responsible for 92.6 ± 5.5 % and 98.6 ± 0.1 % of the total liver uptake of both ligands, respectively. The non-parenchymal cells accounted only for about 7.4 % and 1.3 % of the liver uptake, respectively.

Table I: Relative contribution of the different liver cell types to the liver uptake of sulfated ^{125}I-tcu-PA and non-sulfated ^{125}I-tcu-PA.

Cell type	sulfated ^{125}I-tcu-PA (%)	non-sulfated ^{125}I-tcu-PA (%)
Parenchymal cells	92.6 ± 5.5	98.7 ± 0.1
Endothelial cells	5.4 ± 3.1	0.4 ± 0.1
Kupffer cells	2.0 ± 0.6	0.9 ± 0.1

The different liver cell types were isolated 10 min after injection of sulfated ^{125}I-tcu-PA and non-sulfated ^{125}I-tcu-PA (1.0 μg/kg body weight). The % of the injected dose/mg cell protein in each cell fraction isolated (not shown) was multiplied by the relative contribution of each cell type to the total liver uptake. Recovery of the injected dose in the different liver cell types was 101.8 ± 4.5 % and 95.4 ± 1.5 % for sulfated ^{125}I-tcu-PA and non-sulfated ^{125}I-tcu-PA, respectively. Data represent the mean of 2 experiments ± the variation of the individual experiments.

In vitro interaction of sulfated tcu-PA and non-sulfated tcu-PA with parenchymal liver cells

Since the parenchymal liver cells were responsible for the majority of the uptake of sulfated tcu-PA and non-sulfated tcu-PA, we studied the mechanism of interaction with freshly isolated parenchymal liver cells. Figure 2 shows that at 1 nM substrate concentration the parenchymal cell association of non-sulfated ^{125}I-tcu-PA was 65.4 ± 5.0 fmol/mg cell protein, while sulfated ^{125}I-tcu-PA showed a 20-fold lower parenchymal cell association (3.3 ± 0.3 fmol/mg cell protein). Competition experiments were performed in order to determine the potential involvement of the ASGPr in the recognition of the ligands since the tcu-PA mixture, not separated into sulfated and non-sulfated tcu-PA did show earlier an interaction with the ASGPr.[18] Displacement studies of non-sulfated ^{125}I-tcu-PA with tcu-PA resulted in an inhibition constant of 88.9 ± 2.1 nM. Taking into account that only 30 % of the unlabelled tcu-PA preparation consists of non-sulfated tcu-PA, a K_i of 26.7 ± 0.6 nM was calculated for the association of non-sulfated tcu-PA to the parenchymal liver cells (figure 2A). ASOR was an efficient competitor for the

binding of non-sulfated ^{125}I-tcu-PA (K_i = 10.0 ± 1.1 nM). On the other hand, the binding of sulfated ^{125}I-tcu-PA to the parenchymal cells could not be competed for by ASOR or tcu-PA (figure 2B).

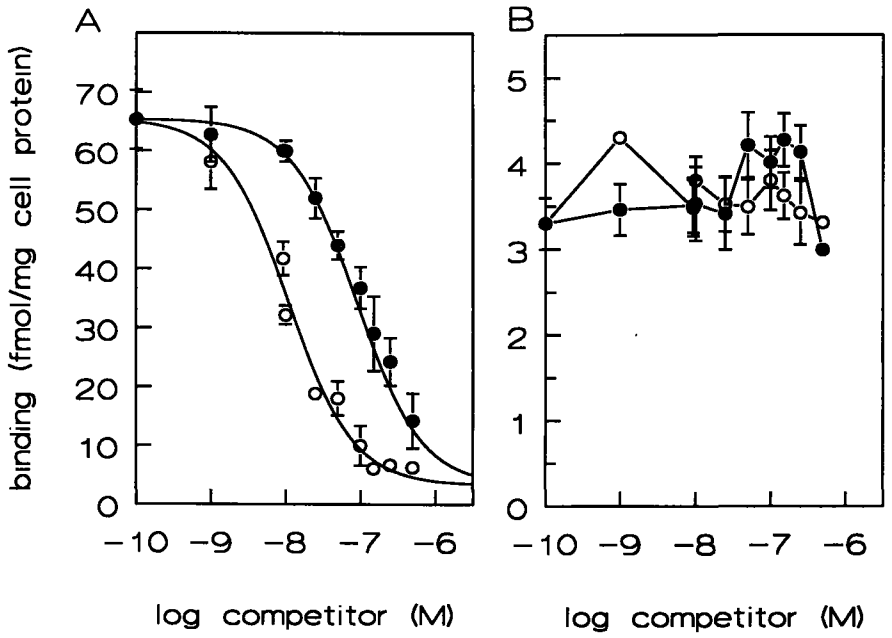

Figure 2. Effect of ASOR and tcu-PA on the binding of sulfated ^{125}I-tcu-PA and non-sulfated ^{125}I-tcu-PA to rat parenchymal liver cells. Rat parenchymal liver cells were incubated for 2 h at 4 °C in the presence of 1 nM non-sulfated ^{125}I-tcu-PA (A) or sulfated ^{125}I-tcu-PA (B) and increasing amounts of unlabelled ASOR (O) or tcu-PA (●). The binding is expressed as fmol protein/mg cell protein and represents the means of 3 experiments ± the S.D.

The different recognition of sulfated tcu-PA and non-sulfated tcu-PA was also studied by determination of the inhibitory effect of these two preparations on the binding of ^{125}I-ASOR[30], a high affinity ligand for the ASGPr (figure 3). It was found that 1000 nM of non-sulfated tcu-PA was able to compete for the binding of ^{125}I-ASOR to the ASGPr for 88 %. On the other hand, sulfated tcu-PA was not able to compete et all for the binding of ^{125}I-ASOR to the ASGPr. Removal of the complex carbohydrate side chain at Asn_{302} of non-sulfated and sulfated tcu-PA by treatment with N-glycosidase F abolished the inhibitory effect of non-sulfated tcu-PA but did not influence the absence of inhibition by sulfated tcu-PA.

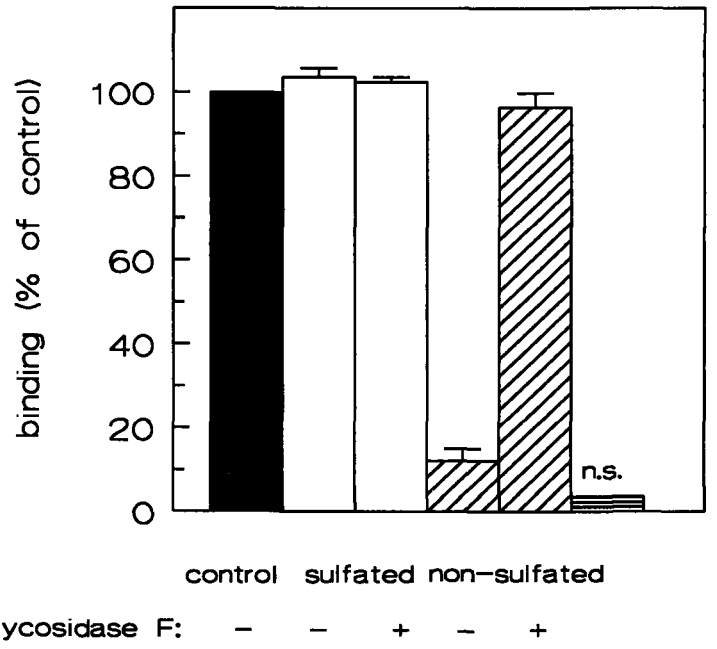

Figure 3. Parenchymal liver cell binding of ^{125}I-ASOR: effect of competition with sulfated and non-sulfated tcu-PA which was treated with or without of N-glycosidase F. Rat parenchymal liver cells were incubated for 2 h at 4 °C in the presence of 1 nM ^{125}I-ASOR (closed bar). The open bar represents the binding of 1 nM ^{125}I-ASOR in the presence of 1000 nM sulfated ^{125}I-tcu-PA with (+) or without (-) a pretreatment of N-glycosidase F. The hatched bar represents the binding of 1 nM ^{125}I-ASOR in the presence of 1000 nM non-sulfated ^{125}I-tcu-PA with (+) or without (-) a pretreatment of N-glycosidase F. The horizontal striped bar (n.s.) represents the non-specific binding (3.6 %). Data are expressed as % of control binding (100 % = 1.35 ± 0.02 pmol/mg cell protein) and represent the means of 4 experiments ± the S.D.

Serum decay and liver uptake of non-sulfated tcu-PA: effect of inhibitors

In order to investigate whether the specificity of the parenchymal liver cell interaction *in vitro* was relevant for its metabolic fate *in vivo*, we injected 1 min prior to injection of non-sulfated ^{125}I-tcu-PA a high amount of ASOR (figure 4A). Pretreatment with ASOR reduced the maximal liver uptake at 10 min after injection for 70 % from 67.6 ± 0.3 % i.d. to 20.6 ± 1.9 % i.d. The clearance of non-sulfated ^{125}I-tcu-PA was reduced 1.9-fold from 2.5 ± 0.1 ml/min for control rats to 1.3 ± 0.1 ml/min for rats pretreated with ASOR.

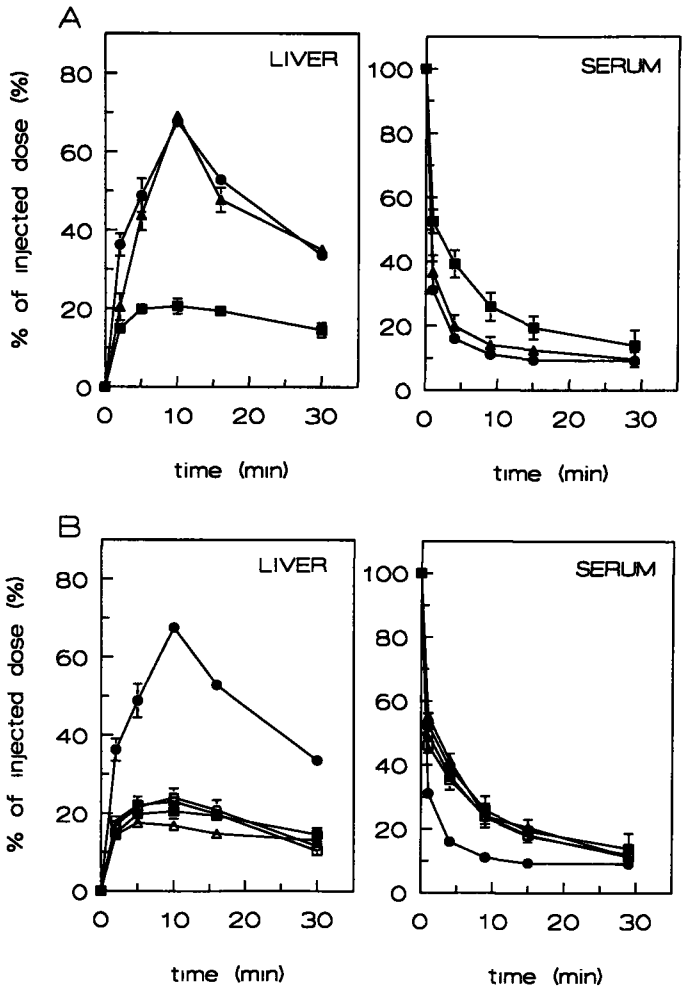

Figure 4. Liver uptake and serum decay of non-sulfated ^{125}I-tcu-PA: effect of inhibitors and/or N-glycosidase F pretreatment. Rats were injected intravenously with (■, figure A, B) or without (●, figure A) a preinjection of 15 mg/kg body weight (b.w.) ASOR 1 min prior to injection of non-sulfated ^{125}I-tcu-PA (1.0 μg/kg b.w.). Other rats were injected with 50 mg/kg b.w. protamine sulfate (▲, figure A) or with N-glycosidase F treated non-sulfated ^{125}I-tcu-PA (O, figure B). One min prior to injection of N-glycosidase F treated non-sulfated ^{125}I-tcu-PA, ASOR (□, figure B) or protamine sulfate (Δ, figure B) were injected. At the indicated times after injection of the radiolabelled ligand, the serum decay and liver uptake were determined. Data are expressed as % of the injected dose and represent the means of 2 experiments ± the variation of the individual experiments. When the variation is not visible, it fell within the symbol size.

As we have recently shown[18], urinary tcu-PA can be recognized by heparan sulfate proteoglycans (HSPGs) on parenchymal liver cells. In order to study the involvement of HSPGs in the recognition of non-sulfated tcu-PA, we injected 1 min prior to injection of non-sulfated ^{125}I-tcu-PA, 50 mg/kg body weight protamine sulfate[31] (figure 4A). No significant effect on the liver uptake and serum decay of non-sulfated tcu-PA was observed.

The role of the ASGPr in the recognition non-sulfated tcu-PA was also indicated by treatment of non-sulfated ^{125}I-tcu-PA with N-glycosidase F. Figure 4B shows that the removal of the carbohydrate from non-sulfated tcu-PA reduces its liver uptake and delays its serum clearance to the same extent as a preinjection of ASOR. As a control for the N-glycosidase F treatment, non-sulfated ^{125}I-tcu-PA was also incubated for 18 hour at 37 °C without N-glycosidase F. After injection no effect on the liver uptake or serum clearance was observed (data not shown). ASOR (15 mg/kg body weight) did not affect the liver uptake and serum clearance of N-glycosidase F treated non-sulfated ^{125}I-tcu-PA (figure 4B). A preinjection of 50 mg/kg body weight protamine sulfate reduced the maximum liver uptake of N-glycosidase F treated non-sulfated ^{125}I-tcu-PA for 30 % (figure 4B, (from 24.1 ± 2.3 % i.d. for N-glycosidase F treated non-sulfated ^{125}I-tcu-PA to 16.8 ± 1.2 % i.d. for N-glycosidase F treated non-sulfated ^{125}I-tcu-PA with a protamine sulfate preinjection)).

Serum decay and liver uptake of sulfated tcu-PA: effect of inhibitors

Since we recently showed that non WFA-Sepharose separated urinary tcu-PA is recognized both by the ASGPr and HSPGs[18] and we just showed that the part of urinary tcu-PA that carries GalNAc terminal carbohydrate chains is recognized by the ASGPr, we studied whether the sulfated tcu-PA is recognized by HSPGs *in vivo*. Therefore, we injected 1 min prior to injection of 1.0 μg/kg body weight sulfated ^{125}I-tcu-PA 50 mg/kg body weight protamine sulfate. Figure 5A shows that the maximal liver uptake at 10 min after injection was decreased 45 % from 33.7 ± 4.1 % i.d. to 18.5 ± 5.4 % i.d. The clearance of sulfated ^{125}I-tcu-PA was reduced 1.6-fold from 1.8 ± 0.1 ml/min for control rats to 1.1 ± 0.1 ml/min for rats pretreated with protamine sulfate.

Although sulfated tcu-PA did not bind to the ASGPr on isolated parenchymal liver cells and in a purified system[19], we unexpectedly observed a 29 % reduction in liver uptake after a preinjection of 15 mg/kg body weight ASOR (figure 5A). The maximal liver uptake was reduced from 33.7 ± 4.1 % i.d. to 23.8 ± 1.6 % i.d. We further investigated this effect by removal of the carbohydrate moiety of sulfated ^{125}I-tcu-PA by treatment with N-glycosidase F. As shown in figure 5B, removal of the carbohydrate side chain of sulfated ^{125}I-tcu-PA reduced the liver uptake to the same extent as sulfated ^{125}I-tcu-PA with a preinjection of ASOR. A preinjection of ASOR had no effect on the liver uptake and serum clearance of N-

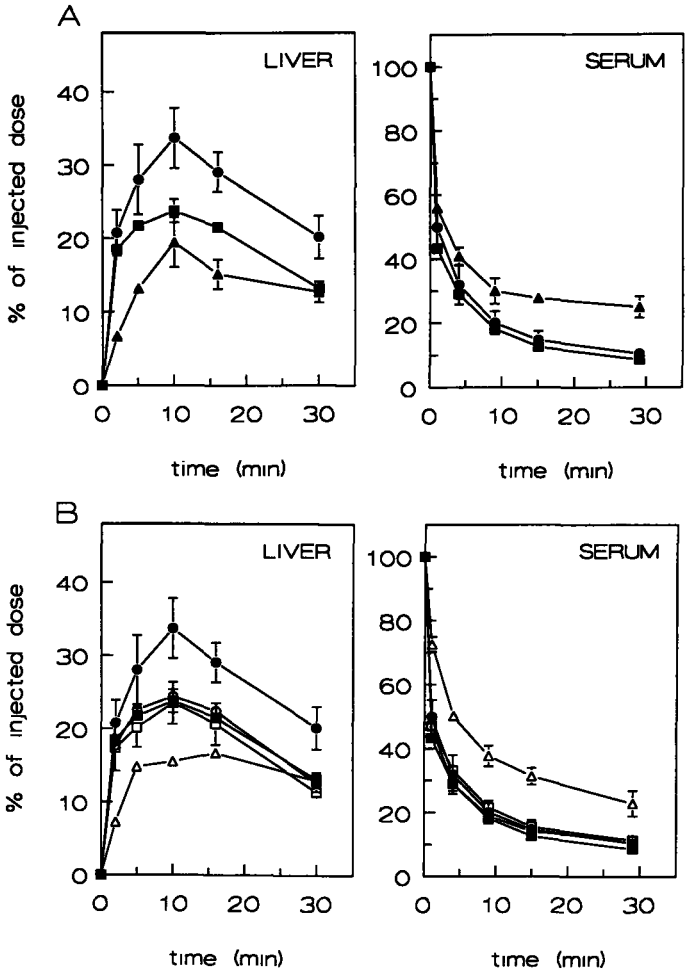

Figure 5. Liver uptake and serum decay of sulfated ^{125}I-tcu-PA: effect of inhibitors and/or N-glycosidase F pretreatment. Rats were injected intravenously with (■, figure A, B) or without (●, figure A) a preinjection of 15 mg/kg body weight (b.w.) ASOR 1 min prior to injection of sulfated ^{125}I-tcu-PA (1.0 μg/kg b.w.). Other rats were injected with 50 mg/kg b.w. protamine sulfate (▲, figure A) or with N-glycosidase F treated sulfated ^{125}I-tcu-PA (O, figure B). One min prior to injection of N-glycosidase F treated sulfated ^{125}I-tcu-PA, ASOR (□, figure B) or protamine sulfate (Δ, figure B) were injected. At the indicated times after injection of the radiolabelled ligand, the serum decay and liver uptake were determined. Data are expressed as % of the injected dose and represent the means of 2 experiments ± the variation of the individual experiments. When the variation is not visible, it fell within the symbol size.

glycosidase F treated sulfated ^{125}I-tcu-PA. Preinjection of protamine sulfate reduced the liver uptake and serum clearance of N-glycosidase F treated sulfated ^{125}I-tcu-PA from 23.8 ± 1.6 % i.d. and 1.7 ± 0.2 ml/min to 15.5 ± 0.9 % i.d. and 0.9 ± 0.2 ml/min (figure 5B).

The residual liver uptake after inhibition of the ASGPr and/or HSPGs is the same value for sulfated and non-sulfated tcu-PA. This indicates that there is a residual liver uptake of 15 % i.d. for sulfated or non-sulfated tcu-PA that is not mediated via the ASGPr or HSPGs.

Organ distribution of sulfated tcu-PA and non sulfated tcu-PA

We determined whether other organs than the liver contributed specifically to the removal of sulfated ^{125}I-tcu-PA and non-sulfated ^{125}I-tcu-PA from the circulation (figure 6). Besides the liver (39.8 ± 3.1 % i.d.), the muscles (5.6 ± 0.1 % i.d.), kidneys (5.6 ± 0.1 % i.d.), skin (9.4 ± 0.2 % i.d.) and spleen (4.1 ± 0.2 % i.d.) contributed to the uptake of sulfated ^{125}I-tcu-PA. The clearance of non-sulfated

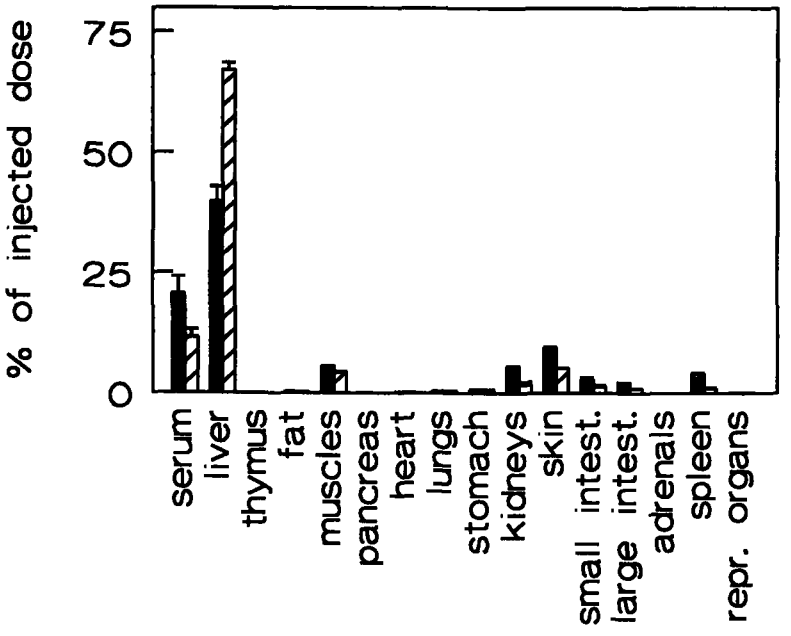

Figure 6. Organ distribution of sulfated ^{125}I-tcu-PA or non-sulfated ^{125}I-tcu-PA. Ten min after injection of the ligands, the amount of radioactivity in the various organs was determined. Recovery of the injected amount of radioactivity in the tissues shown was 91.9 ± 2.3 % for sulfated ^{125}I-tcu-PA (closed bars) and 94.7 ± 3.1 % for non-sulfated ^{125}I-tcu-PA (hatched bars). Data expressed as % of the injected dose are mean ± S.D. (n = 3).

^{125}I-tcu-PA was mediated besides by the liver (67.1 ± 1.5 % i.d.), by the muscles (4.3 ± 0.1 % i.d.), kidneys (1.7 ± 0.7 % i.d.), skin (5.2 ± 0.1 % i.d.) and spleen (1.0 ± 0.1 % i.d.) The contribution of the extrahepatic organs in the clearance was higher for sulfated ^{125}I-tcu-PA than for non-sulfated ^{125}I-tcu-PA (24.7 and 12.2 %, respectively).

DISCUSSION

Recently we reported that urinary tcu-PA is recognized within the rat liver by the asialoglycoprotein receptor (ASGPr) and by heparan sulfate proteoglycans (HSPGs) on parenchymal cells.[18] As is shown by Bergwerff *et al.*[16], the carbohydrate moiety of the urinary tcu-PA preparation is a mixture of sulfated and non-sulfated GalNAcß1-4GlcNAcß1-2Man terminal bi- and triantennary glycans. Therefore we were interested whether sulfation prevents recognition of urinary tcu-PA by the ASGPr and results in interaction with HSPGs. The tcu-PA preparation was separated by means of a Wisteria floribunda agglutinin Sepharose column to obtain a sulfated tcu-PA (70 % of the preparation) and a non-sulfated tcu-PA fraction (30 % of the preparation).[19] The present study shows that non-sulfated tcu-PA is recognized by the ASGPr and that sulfated tcu-PA is recognized by HSPGs.

Our *in vivo* studies demonstrated that non-sulfated tcu-PA is taken up by the liver for 67.6 ± 0.3 % i.d. and that sulfated tcu-PA is taken up by the liver upto 33.7 ± 4.1 % i.d. Non-sulfated tcu-PA is cleared 1.4-fold faster from the circulation than sulfated tcu-PA (2.5 ± 0.1 ml/min and 1.8 ± 0.1 ml/min, respectively). Both tcu-PA preparations are almost exclusively taken up by the parenchymal liver cells, which is in accordance with our previous publication on the liver uptake of the urinary tcu-PA mixture.[18]

Besides the liver, the kidneys, muscles, skin and spleen contribute to the clearance of both sulfated and non-sulfated tcu-PA. The contribution of these organs to the clearance of sulfated tcu-PA was 2-fold higher (24.7 % i.d. at 10 min after injection) than the contribution in the clearance of non-sulfated tcu-PA (12.2 % i.d.). This may explain that although the liver uptake of sulfated tcu-PA is 2-fold reduced compared to non-sulfated tcu-PA, sulfated tcu-PA is still rapidly removed from the circulation (only 1.4-fold reduced clearance).

Binding studies with freshly isolated parenchymal liver cells showed that non-sulfated tcu-PA binds to the ASGPr with high affinity (K_i = 26.7 ± 0.6 nM) and that the binding could be completely inhibited by ASOR (a high affinity ligand for the ASGPr, (K_i = 10.0 ± 1.1 nM)). Recognition of non-sulfated tcu-PA by the ASGPr *in vitro* was further illustrated by the 88 % inhibition of the binding of ^{125}I-ASOR by non-sulfated tcu-PA and by the inability of N-glycosidase F treated non-sulfated tcu-PA to compete for the binding of ^{125}I-ASOR to the ASGPr. Sulfated tcu-PA on the other hand, showed a 20-fold reduced parenchymal liver cell

binding (3.3 ± 0.3 fmol/mg cell protein v.s. 65.4 ± 5.0 fmol/mg cell protein for sulfated tcu-PA and non-sulfated tcu-PA, respectively). The binding of sulfated tcu-PA could not be competed for by ASOR or tcu-PA. This points to the presence of a binding site for sulfated tcu-PA that differs from the ASGPr. HSPGs are suitable candidates but it is most likely that due to the isolation procedure of the parenchymal liver cells in which collagenase is used, most proteoglycans are removed from the cell surface. This explains why little tcu-PA binds to the parenchymal liver cells and why tcu-PA was not able to compete for the binding of sulfated ^{125}I-tcu-PA. LRP, which is able to bind recombinant non-glycosylated scu-PA[20,24,32] did not contribute to the uptake of sulfated and non-sulfated tcu-PA since GST-RAP (a protein that is capable to inhibit the binding of all known ligands to LRP) did not affect their binding (data not shown). GST-RAP did also not influence the binding of the urinary tcu-PA mixture.[18]

In order to study *in vivo* the relevance of the observed differences in recognition, we preinjected ASOR 1 min prior to injection of non-sulfated tcu-PA or removed the carbohydrate moiety of non-sulfated tcu-PA by incubation with N-glycosidase F. Both procedures resulted in a 70 % reduced maximal liver uptake and a 1.9-fold fold delayed serum clearance. The involvement of negatively charged HSPGs in the recognition of non-sulfated tcu-PA by the liver was studied by a preinjection of the HSPG antagonist protamine sulfate, which specifically inhibited the binding of Tissue Factor Pathway Inhibitor to mouse liver HSPGs *in vivo*.[31] No clear effect was observed on the liver uptake and serum decay of non-sulfated tcu-PA. Due to the heparin binding capacity of u-PA[33,34] and the net positive charge of u-PA, recognition by the negatively charged proteoglycans was expected, but not clearly observed until the carbohydrate moiety of non-sulfated tcu-PA was removed (the liver uptake of N-glycosidase F treated non-sulfated tcu-PA reduced from 24.1 % i.d. to 16.8 % i.d. upon a preinjection of protamine sulfate). This can be explained by the fact that the GalNAc-terminal glycans of the non-sulfated tcu-PA fraction are recognized by the ASGPr with high affinity and account for at least 70 % of the liver uptake *in vivo*. The interaction of non-sulfated tcu-PA with the HSPGs accounts only for about 5 % of the liver uptake *in vivo*. Only after disturbance of the interaction with the preferred interaction site (ASGPr) the recognition of tcu-PA by HSPGs becomes evident. This is confirmed by the results obtained with sulfated tcu-PA.

The involvement of HSPGs in the binding of sulfated tcu-PA was shown by a 45 % reduced maximal liver uptake (from 33.7 ± 4.1 % i.d. (control) to 18.5 ± 5.4 % i.d. (protamine sulfate)) and a 1.6-fold reduced clearance upon a preinjection of protamine sulfate. Unexpectedly a preinjection of ASOR reduced the maximal liver uptake by 29 % from 33.7 ± 4.1 % i.d. to 23.8 ± 1.6 % i.d. This suggests that sulfated tcu-PA is recognized by the ASGPr *in vivo* although no evidence for this interaction was found in a purified ASGPr system[19] or on parenchymal liver cells bearing the ASGPr (no competition of ASOR for the binding of sulfated ^{125}I-tcu-PA

and no inhibition of the binding of ^{125}I-ASOR by sulfated tcu-PA). When the carbohydrate moiety of sulfated ^{125}I-tcu-PA was removed by treatment with N-glycosidase F the liver uptake was reduced to the same extent as when ASOR was preinjected. This confirms that the observed interaction with the ASGPr indeed was mediated via the carbohydrate moiety of tcu-PA. Inhibition of the interaction with HSPGs by a preinjection of protamine sulfate reduced the liver uptake of N-glycosidase F treated sulfated tcu-PA to 15.5 ± 0.9 % i.d. and delayed the clearance to 0.9 ± 0.2 ml/min. A residual liver uptake of about 15 % i.d. of sulfated or non-sulfated tcu-PA is left that is not mediated via the ASGPr or HSPGs. This is the same value as described before for the urinary tcu-PA mixture.[18] As suggested before probably other proteoglycans in the liver may be involved in this association.

An explanation for the observed partial recognition of sulfated tcu-PA by the ASGPr *in vivo* may be the presence of sulfatases such as arylsulfatase[35] in the blood circulation. Our data indicate that it is possible that such a mechanism plays a role *in vivo* since in the first 5 minutes after injection of sulfated tcu-PA with or without a preinjection of ASOR no effect on the liver uptake is observed (figure 5A). Five minutes after injection ASOR starts to inhibit the liver uptake of sulfated tcu-PA. This may be explained by the fact that after injection sulfatases start to remove SO_4 from sulfated ^{125}I-tcu-PA. In the time course of the experiment the amount of non-sulfated ^{125}I-tcu-PA starts to increase and uptake by the ASGPr is accomplished. An indication for the recognition of sulfated tcu-PA by the SO_4-GalNAcß1-4GlcNAcß1-2Man recognizing receptor on Kupffer cells[36] was not found since the contribution of the Kupffer cells in the recognition of sulfated tcu-PA was only 2 %.

In conclusion, this study shows that sulfation of the GalNAc terminal bi- and triantennary glycans of urinary tcu-PA determines the receptor specificity of the hepatic parenchymal cell recognition. Non-sulfated GalNAc-terminal tcu-PA is predominantly recognized by the asialoglycoprotein receptor. Sulfation of GalNAc prevents the interaction with the asialoglycoprotein receptor and results in an interaction with heparan sulfate proteoglycans in the liver and to a lesser extent in the kidneys and skin. The dissection of the recognition sites of glycosylated u-PA in the liver provides evidence that regulation of the sulfation of u-PA might be an important factor in its mechanism of clearance. Thrombolytic therapy with urinary tcu-PA may be improved by the addition of an asialoglycoprotein receptor antagonist such as ASOR or the at our lab developed high affinity cluster galactoside TrisGal(20Å).[37] The recognition of the non-sulfated tcu-PA fraction of the urinary tcu-PA preparation by the asialoglycoprotein receptor will be abolished and as a consequence of this more tcu-PA will be available in the circulation to dissolve the stopped up blood vessel. Isolation of the non-sulfated tcu-PA fraction from the preparation and addition of the aforementioned asialoglycoprotein receptor antagonist TrisGal(20Å) together with non-sulfated tcu-PA may improve

thrombolytic therapy probably more.

ACKNOWLEDGEMENT

This study was supported by the Netherlands Heart Foundation (grant 92.139).

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Chapter 7

The effect of complex formation of urinary urokinase-type plasminogen activator and Plasminogen Activator Inhibitor type-1 on the liver uptake and serum clearance in rats

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SUMMARY

The Low Density Lipoprotein Receptor-related Protein (LRP) mediates the cellular internalization of urokinase-type (u-PA) and tissue-type (t-PA) plasminogen activator with lower affinity than plasminogen activator complexed with their inhibitor plasminogen activator inhibitor type 1 (PAI-1). Recently we have showed that urinary tcu-PA is recognized by the asialoglycoprotein receptor (ASGPr) and by heparan sulfate proteoglycans (HSPGs) but not by LRP on rat parenchymal liver cells although these cells express LRP. Therefore, we were interested whether complex formation of urinary tcu-PA with PAI-1 results in the induction of recognition of the complex by LRP.

Complex formation of tcu-PA with PAI-1 resulted in a 2-fold higher maximum liver uptake at 10 min after injection and concomitantly a 1.6-fold increased serum clearance. A preinjection of the LRP inhibitor GST-RAP reduced the maximal liver uptake of ^{125}I-tcu-PA:PAI-1 from 79.1 ± 2.4 % i.d. to 36.7 ± 1.7 % i.d. Concurrently, the serum clearance was delayed from 2.5 ± 0.2 ml/min to 1.1 ± 0.2 ml/min. Recognition of tcu-PA:PAI-1 via its carbohydrate moiety by the ASGPr was shown by the inhibitory effect of ASOR on the maximum liver uptake (from 79.1 ± 2.4 % i.d. to 65.0 ± 2.8 % i.d.) and reduced serum clearance to 1.8 ± 0.2 ml/min. The involvement of HSPGs was shown by a reduction in the liver uptake to 57.4 ± 1.4 % i.d. after injection of protamine sulfate.

The role of the lysine 69, 80 and 88 residues in the heparin binding domain of PAI-1 in the recognition of the tcu-PA:PAI-1 complex by LRP was studied by the use of the recently described lysine to alanine mutants of PAI-1. Tcu-PA:(K69A,K80A,K88A)-PAI-1 and tcu-PA:(K69A)-PAI-1 showed a 1.6-fold reduced maximal liver uptake and a 1.6-fold delayed serum clearance compared to tcu-PA:PAI-1. A preinjection of GST-RAP reduced the liver uptake of tcu-PA:(K69A,K80A,K88A)-PAI-1 and tcu-PA:(K69A)-PAI-1 to 30 % i.d. and delayed the serum clearance. Since the liver uptake of tcu-PA:(K69A,K80A,K88A)-PAI-1 and tcu-PA:(K69A)-PAI-1 is reduced to a larger extent by the preinjection of GST-RAP than the maximum liver uptake of uncomplexed tcu-PA (40.8 % i.d.) we suggest that due to the complex formation of tcu-PA with PAI-1 cryptic binding sites not only as recently described within PAI-1 but also in urinary tcu-PA are exposed upon complex formation that mediate the binding of tcu-PA:PAI-1 to LRP. Similar results for PAI-1 were obtained when t-PA was complexed with PAI-1 or the PAI-1 mutants.

In conclusion, this study shows that complex formation of urinary tcu-PA with its physiological inhibitor PAI-1 results in the initiation of recognition of the tcu-PA:PAI-1 complex by LRP. Due to the complex formation cryptic LRP binding sites in PAI-1 (Lysine 69) and probably in tcu-PA are exposed that are responsible for the recognition of tcu-PA:PAI-1 by LRP.

INTRODUCTION

Urokinase-type plasminogen activator (u-PA) is a highly specific serine protease that catalyses the conversion of the zymogen plasminogen into plasmin. Because of its capacity to degrade fibrin via plasminogen activation, u-PA is an attractive thrombolytic agent. The efficacy of u-PA in thrombolytic therapy is restricted by its rapid removal from the circulation with an average half-life ranging from 1 minute for rats to 8 minutes for men.[1-4] The liver and to a lesser extent the kidneys have been identified to play a major role in the uptake of both high molecular weight (HMW) and low molecular weight (LMW) u-PA in monkeys, rabbits and rats.[5-7] Urokinase is produced as a single chain protein (scu-PA)[8] with a molecular mass of 55,000 Da (= HMW-scu-PA) by e.g. cultured kidney cells[9,10], tumor cells[11] and fibroblasts.[12] Proteolytic cleavage of scu-PA between Lys_{158} and Ile_{159} by plasmin or kallikrein results in the conversion into an active two-chain form (tcu-PA). The action of HMW-tcu-PA can be inactivated by the 50,000 kDa Plasminogen Activator Inhibitor type 1 (PAI-1), which results in the formation of an equimolar u-PA:PAI-1 complex.[13]

When expressed in eukaryotic cells, u-PA is secreted as a glycoprotein with an O-linked α-fucose at Thr_{18}[14,15] and a complex N-linked oligosaccharide at Asn_{302}.[16,17] Recently we showed that glycosylated u-PA obtained from human urine is recognized by the asialoglycoprotein receptor (ASGPr) and heparan sulfate proteoglycans (HSPGs) on rat parenchymal liver cells.[4] Furthermore, we showed that sulfation of the GalNAc terminal bi- and triantennary glycans of u-PA prevents the interaction with the asialoglycoprotein receptor and results in the interaction with HSPGs.[18]

It has been shown by several groups that both u-PA[3,19,20] and u-PA:PAI-1 complexes[21,22] are recognized by LRP. The u-PA:PAI-1 complex is recognized by LRP with higher affinity than uncomplexed scu-PA or tcu-PA.[19,20] Therefore, LRP is thought to be a receptor for u-PA:PAI-1 complexes rather than for the free components. It was also shown that LRP recognizes u-PA:PAI-1 complexes both via sites within u-PA and PAI-1.[20] The higher affinity for the u-PA:PAI-1 complexes as compared to the free components may result from the cooperative binding of multiple sites within u-PA and PAI-1 or from novel cryptic binding sites that become exposed on u-PA and/or PAI-1 upon complex formation.

PAI-1 contains a heparin binding domain[23,24] that may be involved in the interaction with LRP since it has been shown that heparin inhibits the binding of various ligands to LRP.[25,26] Ehrlich *et al.*[24] have shown that the positively-charged Lysine 65, 69, 80 and 88 and Arginine 76 residues within the heparin binding domain of PAI-1 are required for the interaction of PAI-1 with heparin.[24] Recently Horn *et al.*[27] showed the presence of a cryptic binding site (Lysine 69) in the heparin binding domain of PAI-1 that only interacts with LRP after complex formation of PAI-1 and t-PA. Substitution of this Lysine 69 by Alanine ((K69A)-

PAI-1) reduced the affinity of the t-PA:(K69A)-PAI-1 complex for LRP 12.4-fold compared to t-PA:PAI-1. It was also shown that Lysine 80 and 88, that are necessary for the binding of PAI-1 to heparin, contribute little to the binding to LRP.

Since glycosylated tcu-PA obtained form human urine is not recognized by LRP[4] we were interested whether complex formation of tcu-PA with PAI-1 results in the induction of recognition of the complex by LRP. In this study we show that complex formation of urinary tcu-PA with PAI-1 resulted in the recognition of the tcu-PA:PAI-1 complex by LRP. Furthermore, due to the complex formation cryptic LRP binding sites within PAI-1 (Lysine 69) and probably in tcu-PA are exposed that are responsible for the recognition of tcu-PA:PAI-1 by LRP.

MATERIALS AND METHODS

Materials

Two-chain urinary urokinase (tcu-PA = "Ukidan") was from Serono (Aubonne, Switzerland). t-PA (Actilyse) was obtained from Genentech. *E.coli* produced recombinant human PAI-1(>90 % active), and the mutants: (K69A)-PAI-1 (30 % active) and (K69A,K80A,K88A)-PAI-1 (39 % active) were produced as described before by I. Horn (Amsterdam, the Netherlands).[27] Bovine serum albumin (BSA fraction V), collagenase type I and IV, neuraminidase (agarose bound), human orosomucoid and protamine sulfate were from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's modified Eagle medium (DMEM) was from Gibco (Irvine, Scotland). ^{125}I (carrier free) in NaOH was obtained from Amersham (Buckinghamshire, United Kingdom). Pronase and ETI Sepharose was obtained from Boehringer Mannheim (Mannheim, Germany). Glu-Gly-Arg Chloromethyl Ketone, dihydrochloride (GGACK) was obtained from Calbiochem (San Diego, USA). Superdex 200 and Lysine Sepharose 4B were obtained from Pharmacia Biotech (Sweden). GST-RAP, a conjugate of the Receptor Associated Protein and glutathione S-transferase[28] was produced in *E.coli* strain DH5α[29] and showed similar inhibitor characteristics as RAP. The pGEX-RAP plasmid was a generous gift from Dr. J. Herz (Dallas, USA). *E.coli* strain DH5α was from Fabagen, (Utrecht, the Netherlands). All other chemicals were of analytical grade.

Protein iodination

tcu-PA and t-PA were iodinated as described earlier[30] using the iodogen method, resulting in a specific radioactivity of 2500-3000 cpm/ng.

Complex formation of ^{125}I-tcu-PA with PAI-1 and purification

10 μg of ^{125}I-tcu-PA and 50 μg PAI-1 (or (K69A)- or (K69A,K80A,K88A)-PAI-1) were incubated for 18 h at 4 °C in 0.1 M Tris.HCl, 0.1 % Trition X-100, pH 8.1. The reaction mixture was diluted to 1 ml with a buffer containing 0.1 M phosphate buffer, 0.5 M NaCl, 0.01 % EDTA, pH 7.0, applied to a 120 ml Superdex 200 column and eluted with the latter buffer. Radioactivity was counted in a gamma-counter in order to detect the elution profile of the ^{125}I-tcu-PA:PAI-1 and ^{125}I-tcu-PA fractions. The purified ^{125}I-tcu-PA:PAI-1 complexes were concentrated by dialysis against 20 % PEG, followed by dialysis against PBS. The purity was checked by 10 % SDS-PAGE.

Complex formation of ^{125}I-t-PA with PAI-1 and purification

10 μg of ^{125}I-t-PA and 50 μg PAI-1 (or (K69A)- or (K69A,K80A,K88A)-PAI-1) were incubated for 18 h at 4 °C in 0.1 M Tris.HCl, 0.1 % Trition X-100, pH 8.1. The reaction mixture was diluted to 2-3 ml with a loading buffer containing 20 mM Tris.HCl, 1 M NaCl and 0.1 % Tween 20, pH 7.6

and applied to a 8 ml Lysine-Sepharose 4B column that was equilibrated with the same buffer. The column was washed with loading buffer for 1 hour. Elution was performed with loading buffer supplemented with 0.2 M 6-aminocaproic acid, pH 7.6. The eluate was applied to 10 ml of ETI-Sepharose (equilibrated with loading buffer) and incubated for 2 h at 4 °C on a roller bank. The ETI-Sepharose was centrifuged at 2000 xg for 5 min and the supernatant containing ^{125}I-t-PA:PAI-1 complexes was stored at 4 °C. Uncomplexed ^{125}I-t-PA was eluted from the ETI-Sepharose by incubation with 0.3 M Arginine, pH 4.5. The purified ^{125}I-t-PA:PAI-1 complexes were concentrated by dialysis against 20 % PEG, followed by dialysis against PBS. The purity was checked by 10 % SDS-PAGE.

Serum decay and tissue uptake

10-12 weeks old male Wistar rats (230-260 g, Broekman Institute BV, Someren, The Netherlands) were anaesthetized by intraperitoneal injection of 15-20 mg of sodium pentobarbital. The abdomen was opened ^{125}I-tcu-PA (≈0.8 μg/kg body weight) that was inactivated by incubation for 10 min at room temperature with 2 μM of GGACK to prevent complex formation with circulating PAI-1 or ^{125}I-tcu-PA complexed with PAI-1, (K69A)-PAI-1, (K69A,K80A,K88A)-PAI-1 (≈1.6 μg/kg body weight), ^{125}I-t-PA (≈0.8 μg/kg body weight) or ^{125}I-t-PA complexed with PAI-1, (K69A)-PAI-1, (K69A,K80A,K88A)-PAI-1 (≈1.6 μg/kg body weight) were injected via the inferior vena cava and at the indicated times liver lobules were tied off and 0.3 ml blood samples were taken from the inferior vena cava and allowed to clot for 30 min. The body temperature of the rats was kept at 36.5-37 °C using an infrared lamp. The respiration was facilitated by administration of carbogen 95/5 (95 % O_2, 5 % CO_2). The blood samples were centrifuged for 2 min at 10,000 xg and 100 μl serum samples were counted for radioactivity. The excised liver lobules (which totally weighed less than 15 % of the liver mass) were weighted and the radioactivity was counted. Total liver uptake was determined by weighing the liver at the end of the experiment. The total amount of radioactivity in the serum and liver lobules was determined as described previously.[3] Plasma clearance curves were analyzed by computerized nonlinear fitting following a biphasic clearance model (t½α, t½ß) using the program Graphpad Prism (Graphpad Software Inc., San Diego, U.S.A.). The clearance was calculated by the formula: clearance = dose/Area Under the $Curve_{0\text{-}29}$. Results are given as mean ± S.D. Asialo-orosomucoid (ASOR) was prepared as described previously by treating orosomucoid with neuraminidase.[4]

Liver cell distribution

Rats were anaesthetized and injected with radiolabelled ligands as described above. After 10 min circulation the liver was perfused at 8 °C with collagenase. Parenchymal (PC), endothelial (EC) and Kupffer cells (KC) were isolated by centrifugation, followed by counterflow centrifugal elutriation at 4 °C as described in detail elsewhere.[3] The contributions of the different liver cell types to the total hepatic uptake of the injected ligand were calculated with the assumption that parenchymal, endothelial and Kupffer cells account for 92.5, 3.3 and 2.5 % of the total liver protein mass, respectively.[3] As found for a number of substrates, no loss of cell-bound label and/or formation of acid-soluble radioactivity occurred during the low temperature cellisolation procedure, leading to a quantitative recovery of radioactivity associated with the isolated liver cells as compared to the total liver association.

RESULTS

Complex formation of ^{125}I-tcu-PA with PAI-1

A mixture of ^{125}I-tcu-PA and PAI-1 incubated for 18 hours at 4 °C to form ^{125}I-tcu-PA:PAI-1 complexes was separated on a Superdex 200 column (figure 1). The purity of the ^{125}I-tcu-PA:PAI-1 complexes was analyzed by SDS-PAGE. The large

first peak contained the ^{125}I-tcu-PA:PAI-1 complex, while the smaller second peak contained uncomplexed ^{125}I-tcu-PA. Similar results were obtained when (K69A,K80A,K88A)-PAI-1 or (K69A)-PAI-1 were used in stead of PAI-1.

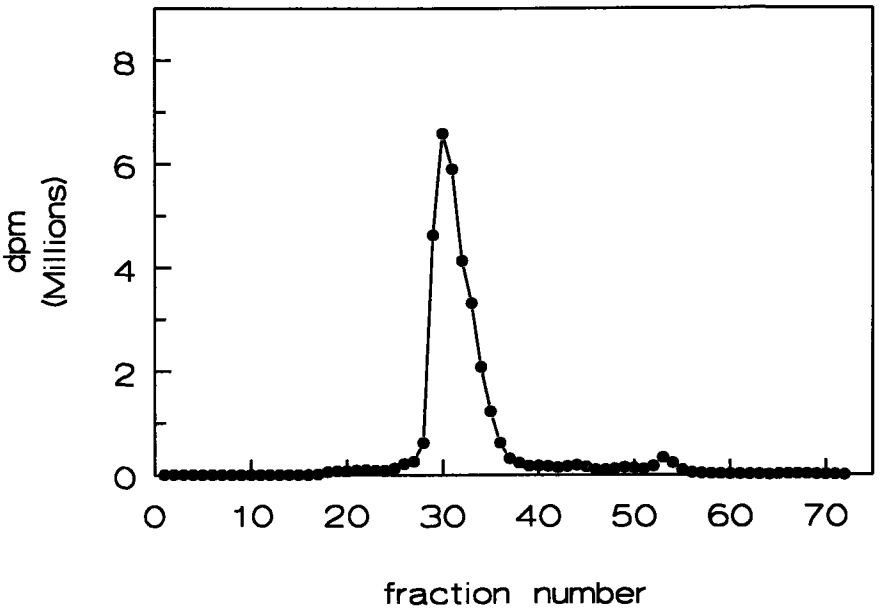

Figure 1. FPLC profile of the purification of ^{125}I-tcu-PA:PAI-1 by Superdex 200. A mixture of ^{125}I-tcu-PA and PAI-1 incubated for 18 hours at 4 °C to obtain ^{125}I-tcu-PA:PAI-1 complexes was separated on a Superdex 200 column. The purity of the ^{125}I-tcu-PA:PAI-1 complexes was analyzed by SDS-PAGE (not shown). The first peak contained the ^{125}I-tcu-PA:PAI-1 complex, while the smaller second peak contained uncomplexed ^{125}I-tcu-PA. Similar results were obtained when (K69A,K80A,K88A)-PAI-1, (K69A)-PAI-1, (K80A,K88A)-PAI-1 were used in stead of PAI-1.

Liver uptake and serum decay of the tcu-PA:PAI-1 complex

After intravenous injection of ^{125}I-tcu-PA:PAI-1 into rats a rapid decay from the blood circulation was observed with a half life of less than 1 min and a clearance of 2.5 ± 0.2 ml/min (figure 2). At 30 min after injection only 8 % of the injected dose was present in the circulation. The rapid clearance of ^{125}I-tcu-PA:PAI-1 was caused by a high uptake in the liver (figure 2). A maximal liver uptake of 79.1 ± 2.4 % of the injected dose (% i.d.) was observed at 10 min after injection. Between 10 and 30 min after injection, the liver associated radioactivity decreased to 19.7 ± 1.8 % i.d., probably due to lysosomal degradation of the ^{125}I-tcu-PA:PAI-1 complex. In comparison to uncomplexed ^{125}I-tcu-PA[4], complex formation of tcu-PA

with PAI-1 resulted in a 2-fold increased liver uptake at 10 min after injection and concurrently in a 1.6-fold increase serum clearance (^{125}I-tcu-PA: liver uptake at 10 min after injection: 40.8 ± 1.6 % i.d., clearance: 1.6 ± 0.1 ml/min).

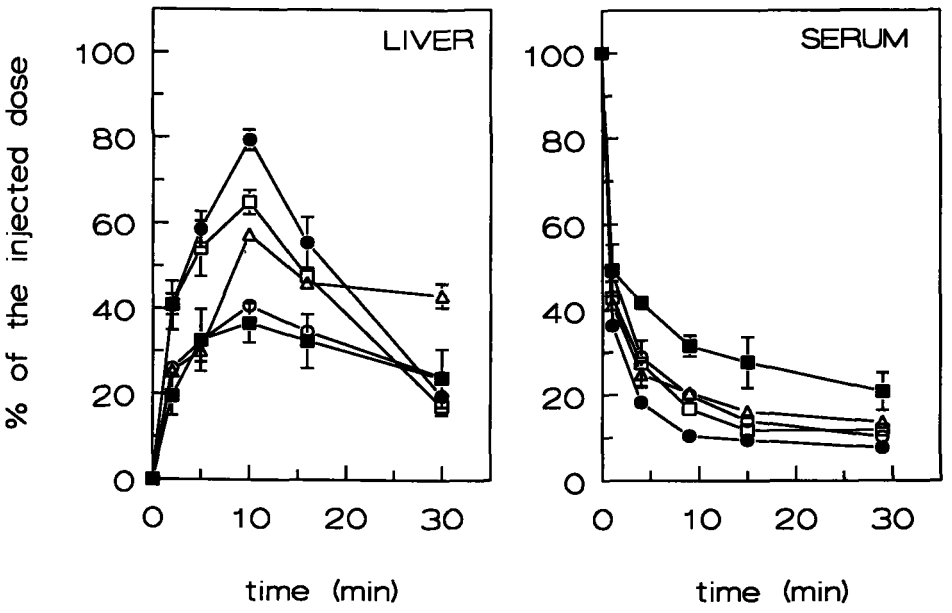

Figure 2. Effect of GST-RAP, ASOR and protamine sulfate on the liver uptake and serum decay of ^{125}I-tcu-PA:PAI-1 complexes. Rats were injected intravenously with ^{125}I-tcu-PA (O)[4] or ^{125}I-tcu-PA:PAI-1 (●). Other rats were injected with 50 mg/kg body weight GST-RAP (■), 15 mg/kg body weight ASOR (□) or 50 mg/kg body weight protamine sulfate (Δ) 1 minute prior to injection of ^{125}I-tcu-PA:PAI-1. At the indicated times after injection of the radiolabelled ligand, the amount of radioactivity in the liver and serum were determined. Data are expressed as % of the injected dose and represent the means of 2-4 experiments ± S.D. When not visible error bars are within symbol size.

To investigate whether the enhanced liver uptake was caused by the recognition of tcu-PA:PAI-1 by LRP, 50 mg/kg body weight GST-RAP (a dose that completely inhibits the recognition of α_2-macroglobulin by LRP in the liver[3]) was injected 1 min prior to the ^{125}I-tcu-PA:PAI-1 complex (figure 2). The liver uptake at 10 min after injection was reduced from 79.1 ± 2.4 % i.d. to 36.7 ± 1.7 % i.d. and the serum clearance was reduced from 2.5 ± 0.2 ml/min to 1.1 ± 0.2 ml/min.

Recently, we showed that uncomplexed urinary tcu-PA is recognized by the asialoglycoprotein receptor (ASGPr).[4] To investigate the effect of complex formation of tcu-PA with PAI-1 on the recognition of the tcu-PA part of the complex by the ASGPr, we injected the antagonist ASOR[31] (15 mg/kg body weight) 1 min prior to injection of the ^{125}I-tcu-PA:PAI-1 complex (figure 2). The maximal liver uptake at 10 min after injection of ^{125}I-tcu-PA:PAI-1 was reduced from 79.5 ± 2.4 % i.d. to 65.0 ± 2.8 % i.d. The blockade of the ASGPr delayed the clearance of ^{125}I-tcu-PA:PAI-1 from 2.5 ± 0.2 ml/min to 1.8 ± 0.2 ml/min.

As we have recently shown[4], urinary tcu-PA is also recognized by heparan sulfate proteoglycans (HSPGs) on parenchymal liver cells. In order to study the effect of complex formation of tcu-PA with PAI-1 on the recognition of the complex by HSPGs, we injected the antagonist protamine sulfate (50 mg/kg body weight) 1 min prior to injection of the ^{125}I-tcu-PA:PAI-1 complex (figure 2). The maximal liver uptake at 10 min after injection of ^{125}I-tcu-PA:PAI-1 was reduced to 57.4 ± 1.4 % i.d. Blockade of the interaction with HSPGs resulted in a 1.5-fold delayed clearance of 1.7 ± 0.2 ml/min.

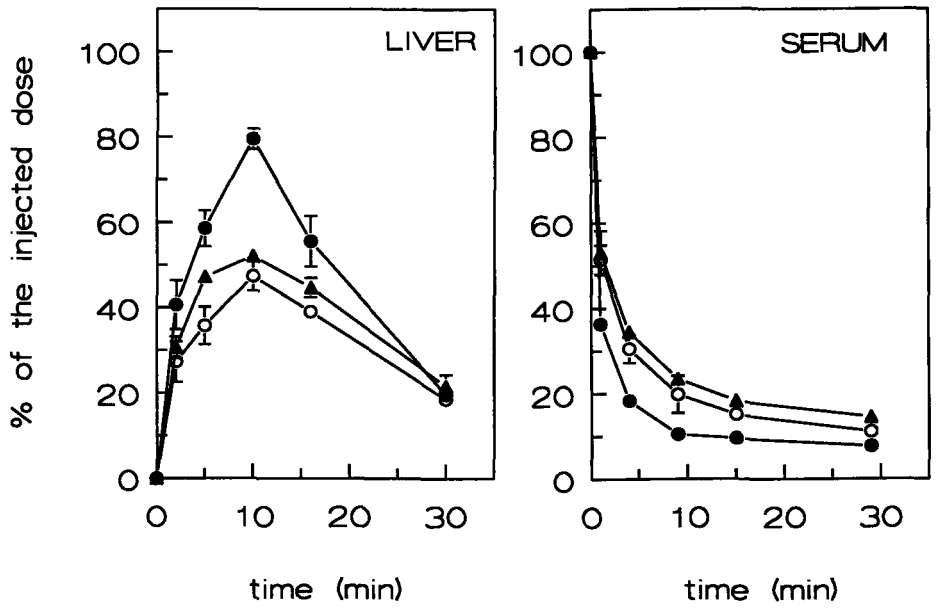

Figure 3. Liver uptake and serum clearance of ^{125}I-tcu-PA complexed with PAI-1, (K69A,K80A,K88A)-PAI-1, (K69A)-PAI-1. Rats were injected intravenously with ^{125}I-tcu-PA complexed with PAI-1 (●), (K69A)-PAI-1 (O) or (K69A,K80A,K88A)-PAI-1 (▲). At the indicated times after injection of the radiolabelled ligand, the amount of radioactivity in the liver and serum was determined. Data are expressed as % of the injected dose and represent the means of 3 experiments ± S.D. When not visible the error bars are within the symbol size.

Liver uptake and serum decay of ^{125}I-tcu-PA complexed with PAI-1 mutants.

The involvement of the heparin binding site of PAI-1 in the observed recognition of the tcu-PA:PAI-1 complex by LRP was studied by the use of the PAI-1 mutants of Horn *et al.*[27] Injection of ^{125}I-tcu-PA:(K69A,K80A,K88A)-PAI-1 resulted in a reduction of the maximal liver uptake from 79.5 ± 2.4 % i.d. (tcu-PA:PAI-1) to 52.1 ± 3.2 % i.d. The serum clearance was delayed from 2.5 ± 0.2 ml/min (tcu-PA:PAI-1) to 1.5 ± 0.1 ml/min (tcu-PA:(K69A,K80A,K88A)-PAI-1) (figure 3). To study whether the observed effects for tcu-PA:(K69A,K80A,K88A)-PAI-1 were mainly caused by Lysine residue 69, ^{125}I-tcu-PA:(K69A)-PAI-1 complexes were prepared and injected into rats. Upon injection of ^{125}I-tcu-PA:(K69A)-PAI-1 a maximal liver uptake of 47.4 ± 3.4 % i.d. at 10 min after injection was observed. A serum clearance of 1.6 ± 0.1 ml/min was determined (figure 3).

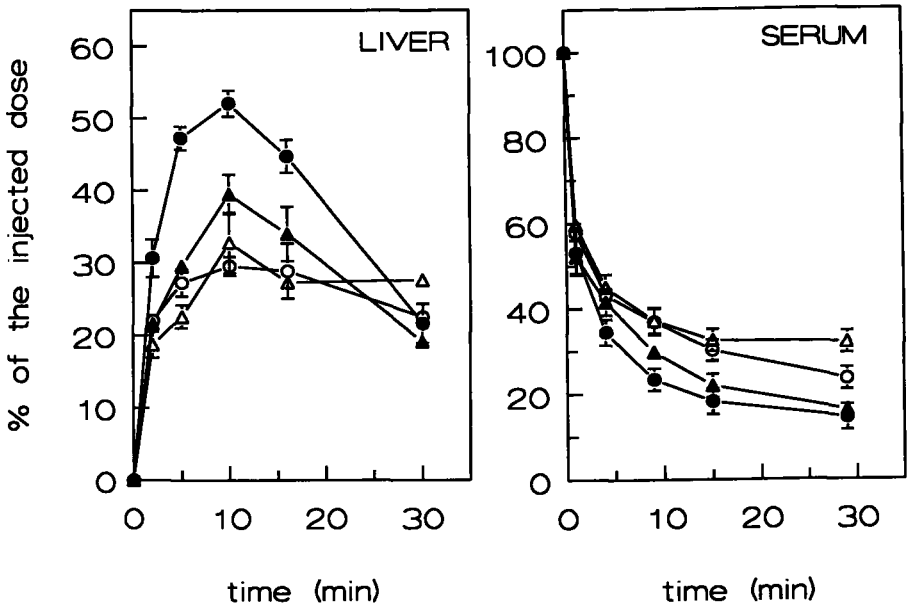

Figure 4. Effect of GST-RAP, ASOR and protamine sulfate on the liver uptake and serum decay of ^{125}I-tcu-PA:(K69A,K80A,K88A)-PAI-1. Rats were injected intravenously without (●) or with 50 mg/kg body weight GST-RAP (O), 15 mg/kg body weight ASOR (▲) or with 50 mg/kg body weight protamine sulfate (Δ) 1 min prior to injection of ^{125}I-tcu-PA:(K69A,K80A,K88A)-PAI-1. At the indicated times after injection of ^{125}I-tcu-PA:(K69A,K80A,K88A)-PAI-1, the amount of radioactivity in the liver and serum were determined. Data are expressed as % of the injected dose and represent the means of 2 experiments ± the variation in the experiments. When not visible error bars are within symbol size.

The contribution of LRP, ASGPR and HSPGs to the liver uptake and serum clearance of ^{125}I-tcu-PA complexed with (K69A,K80A,K88A)-PAI-1 and (K69A)-PAI-1 was determined. Preinjection of ASOR reduced the maximal liver uptake of ^{125}I-tcu-PA:(K69A,K80A,K88A)-PAI-1 from 52.1 ± 3.2 % to 39.5 ± 2.7 % i.d. The serum clearance was slightly delayed from 1.5 ± 0.1 ml/min to 1.3 ± 0.1 ml/min (figure 4). A preinjection of GST-RAP reduced the maximal liver uptake at 10 min after injection of ^{125}I-tcu-PA:(K69A,K80A,K88A)-PAI-1 to 29.6 ± 1.3 % i.d and delayed the serum clearance to 1.0 ± 0.1 ml/min (figure 4). Protamine sulfate reduced the maximum liver uptake at 10 min after injection to 32.8 ± 1.6 % i.d. and delayed the serum clearance to 0.8 ± 0.1 ml/min (figure 4).

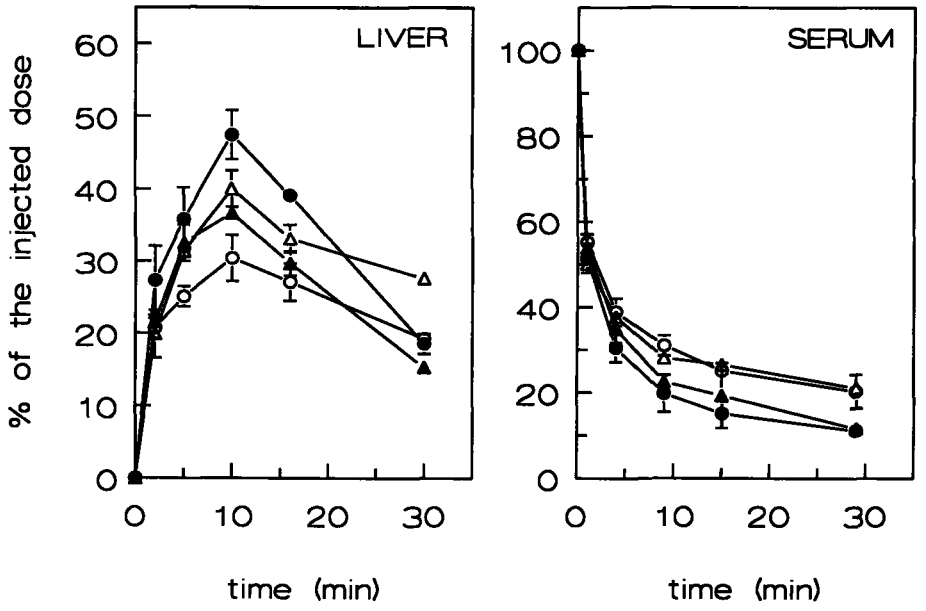

Figure 5. Effect of GST-RAP, ASOR and protamine sulfate on the liver uptake and serum decay of ^{125}I-tcu-PA:(K69A)-PAI-1. Rats were injected intravenously without (●) or with 50 mg/kg body weight GST-RAP (O), 15 mg/kg body weight ASOR (▲) or with 50 mg/kg protamine sulfate (Δ) 1 min prior to injection of ^{125}I-tcu-PA:(K69A)-PAI-1. At the indicated times after injection of ^{125}I-tcu-PA:(K69A)-PAI-1, the amount of radioactivity in the liver and serum were determined. Data are expressed as % of the injected dose and represent the means of 2 experiments ± the variation in the experiments. When not visible error bars are within symbol size.

Blockade of the interaction of ^{125}I-tcu-PA:(K69A)-PAI-1 with the ASGPr by ASOR reduced the maximal liver uptake from 47.4 ± 3.4 % to 36.6 ± 1.1 % i.d. The serum clearance was slightly reduced from 1.6 ± 0.1 ml/min to 1.5 ± 0.2 ml/min

(figure 5). A preinjection of GST-RAP reduced the maximal liver uptake at 10 min after injection of ^{125}I-tcu-PA:(K69A)-PAI-1 to 30.4 ± 3.2 % i.d and delayed the serum clearance to 1.2 ± 0.1 ml/min (figure 5). Protamine sulfate reduced the maximal liver uptake to 40.0 ± 2.5 % i.d. and delayed the serum clearance to 1.3 ± 0.1 ml/min (figure 5).

Liver cell distribution of tcu-PA:PAI-1 and tcu-PA:(K69A,K80A,K88A)-PAI-1

We recently showed that the parenchymal liver cells were responsible for 94.5 ± 0.1 % of the liver uptake of tcu-PA.[4] To study whether complex formation changed the distribution of the complex over the various liver cells, we isolated parenchymal, endothelial and Kupffer cells at 10 min after injection of ^{125}I-tcu-PA:PAI-1 or ^{125}I-tcu-PA:(K69A,K80A,K88A)-PAI-1. Table I shows that compared to uncomplexed tcu-PA the parenchymal cells still were the major liver cell type responsible for the uptake of tcu-PA:PAI-1 or tcu-PA:(K69A,K80A,K88A)-PAI-1 (88.2 ± 0.2 % and 86.1 ± 0.1 %, respectively). The endothelial and Kupffer cells contributed to a somewhat larger extend to the liver uptake of tcu-PA:PAI-1 or tcu-PA:(K69A,K80A,K88A)-PAI-1 compared to tcu-PA (11.8 % and 13.9 % compared to 5.8 %, respectively). The slight induction in contribution of the non-parenchymal liver cells is mainly caused by a 2.5 and 3.2-fold increased recognition of the complexes by the liver endothelial cells, respectively (table I).

Table I. Relative contribution of the different liver cell types to the liver uptake of tcu-PA, tcu-PA:PAI-1 and tcu-PA:(K69A,K80A,K88A)-PAI-1.

Cell type	tcu-PA[4] (%)	tcu-PA:PAI-1 (%)	tcu-PA:(K69A,K80A,K88A)-PAI-1 (%)
Parenchymal cells	94.5 ± 0.1	88.2 ± 0.2	86.1 ± 0.1
Endothelial cells	3.1 ± 0.7	7.9 ± 1.8	9.9 ± 0.1
Kupffer cells	2.7 ± 0.7	3.9 ± 2.0	4.0 ± 0.1

Different liver cell types were isolated 10 min after injection of GGACK-treated ^{125}I-tcu-PA[4], ^{125}I-tcu-PA:PAI-1 or ^{125}I-tcu-PA:(K69A,K80A,K88A)-PAI-1. The % of the injected dose/mg cell protein in each cell fraction isolated (not shown) was multiplied by the relative contribution of each cell type to obtain total liver uptake. Recovery of the injected dose in the different liver cell types was 101.8 ± 4.3 % for ^{125}I-tcu-PA, 98.2 ± 1.4 % for ^{125}I-tcu-PA:PAI-1 and 97.7 ± 0.4 % for ^{125}I-tcu-PA:(K69A,K80A,K88A)-PAI-1. Data represent the mean of 2 experiments ± the variation of the individual experiments.

Relative contribution of the various receptors to the liver uptake of tcu-PA, tcu-PA:PAI-1, and tcu-PA:(K69A,K80A,K88A)-PAI-1 and tcu-PA:(K69A)-PAI-1

The contribution of LRP, ASGPr and HSPGs to the maximum liver uptake at 10 min after injection of ^{125}I-tcu-PA, ^{125}I-tcu-PA:PAI-1, ^{125}I-tcu-PA:(K69A,K80A, K88A)-PAI-1 and ^{125}I-tcu-PA:(K69A)-PAI-1 was determined by calculating the amount of ^{125}I-tcu-PA/complex that was sensitive to inhibition by GST-RAP, ASOR or protamine sulfate depicted in % i.d. in figure 2-5 in fmol. As previously shown[4], no contribution of LRP was determined for the liver recognition of tcu-PA (0 %, table II), the ASGPr accounted for 42 % and HSPGs accounted for 14 % to the liver recognition of tcu-PA (table II). Complex formation of tcu-PA with PAI-1 resulted in the relative contribution of LRP, ASGPr and HSPGs of 54, 18 and 27 %, respectively (table II). For tcu-PA:(K69A,K80A,K88A)-PAI-1 the relative contribution of LRP, ASGPr and HSPGs was determined to be 43, 24 and 37 %. The relative contribution of LRP, ASGPr and HSPGs for tcu-PA:(K69A)-PAI-1 was determined to be 36, 23 and 16 % (table II).

Liver uptake and serum decay of ^{125}I-t-PA complexed with PAI-1, (K69A,K80A,K88A)-PAI-1 or (K69A)-PAI-1 mutants

In order to determine whether the observed effects in recognition by LRP after complex formation of tcu-PA with the PAI-1 mutants were comparable for t-PA complexed with the PAI-1 mutants the liver uptake and serum decay of ^{125}I-t-PA complexed with PAI-1, (K69A,K80A,K88A)-PAI-1 or (K69A)-PAI-1 was studied. ^{125}I-t-PA was rapidly cleared from the circulation with a half-life of less than one min (figure 6). At 10 min after injection a maximal liver uptake of 76.8 ± 3.8 % i.d. was reached (figure 6). Complex formation of ^{125}I-t-PA with PAI-1 resulted in an increased maximal liver uptake from 76.8 ± 3.8 % i.d. to 93.6 ± 3.0 % i.d. at 10 min after injection. No clear difference in the clearance of ^{125}I-t-PA and ^{125}I-t-PA:PAI-1 was observed (4.2 ± 0.2 ml/min and 3.8 ± 0.3 ml/min for ^{125}I-t-PA and ^{125}I-t-PA:PAI-1, respectively). Upon injection of ^{125}I-t-PA:(K69A,K80A,K88A)-PAI-1 a maximal liver uptake of 80.0 ± 2.6 % i.d. at 10 min after injection (figure 6) and a serum clearance of 3.7 ± 0.2 ml/min was determined. Injection of ^{125}I-t-PA:(K69A)-PAI-1 resulted in a maximal liver uptake of 79.1 ± 3.8 % i.d and a serum clearance of 3.3 ± 0.3 ml/min.

Table II. Contribution of LRP, ASGPr and HSPGs to the liver uptake of tcu-PA, tcu-PA:PAI-1, tcu-PA:(K69A,K80A,K88A)-PAI-1 and tcu-PA:(K69A)-PAI-1.

	uptake (fmol)	(%)
tcu-PA		
total liver uptake	1484	(100 %)
LRP	0	(0 %)
ASGPr	622	(42 %)
HSPGs	204	(14 %)
tcu-PA:PAI-1		
total liver uptake	3013	(100 %)
LRP	1615	(54 %)
ASGPr	537	(18 %)
HSPGs	827	(27 %)
tcu-PA:(K69A,K80A,K88A)-PAI-1		
total liver uptake	1985	(100%)
LRP	857	(43 %)
ASGPr	480	(24 %)
HSPGs	735	(37 %)
tcu-PA:(K69A)-PAI-1		
total liver uptake	1806	(100%)
LRP	648	(36 %)
ASGPr	441	(23 %)
HSPGs	282	(16 %)

The contribution of the Low Density Lipoprotein Receptor-related Protein (LRP), asialoglycoprotein receptor (ASGPr) and heparan sulfate proteoglycans (HSPGs) to the maximum liver uptake at 10 min after injection of tcu-PA, tcu-PA:PAI-1, tcu-PA:(K69A,K80A,K88A)-PAI-1 and tcu-PA:(K69A)-PAI-1 was determined by calculating the amount of tcu-PA/complex that was sensitive to inhibition by GST-RAP (represents the interaction with LRP), ASOR (represents ASGPr interaction) or protamine sulfate (represents the interaction with HSPGs) in fmol. (%) indicates the relative contribution of the receptors in the liver uptake for the described ligand. The maximum liver uptake values at 10 min after injection were obtained from figure 2-5.

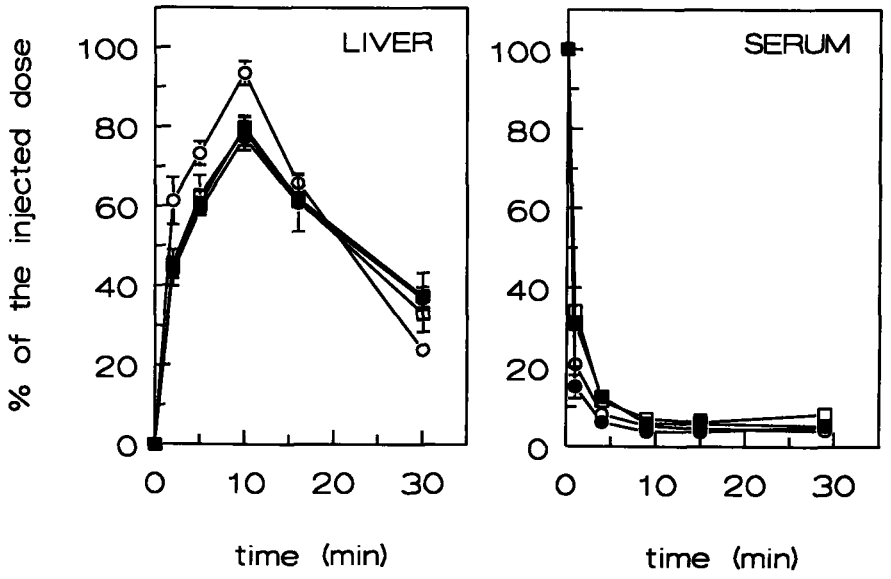

Figure 6. Liver uptake and serum decay of ^{125}I-t-PA complexed with PAI-1 (K69A,K80A,K88A)-PAI-1 or (K69A)-PAI-1 mutants. Rats were injected intravenously with ^{125}I-t-PA (●), ^{125}I-t-PA:PAI-1 (O) ^{125}I-t-PA:(K69A,K80A,K88A)-PAI-1 (■) or ^{125}I-tcu-PA:(K69A)-PAI-1 (□). At the indicated times after injection of the radiolabelled ligands, the amount of radioactivity in the liver and serum were determined. Data are expressed as % of the injected dose and represent the means of 2-4 experiments ± S.D. When not visible error bars are within symbol size.

DISCUSSION

It has been shown that both u-PA[3,19,20] and u-PA:PAI-1 complexes[21,22] are recognized by LRP with different affinity. U-PA:PAI-1 complexes are recognized with higher affinity than uncomplexed scu-PA or tcu-PA.[19,20] Therefore, LRP is thought to be a receptor for u-PA:PAI-1 complexes rather than for the free components. It was also shown that LRP recognizes u-PA:PAI-1 complexes both via sites within u-PA and PAI-1.[20] The higher affinity for the u-PA:PAI-1 complexes compared to the free components could be due to the cooperative binding of multiple sites within both the amino terminal fragment and protease domain of u-PA and PAI-1 or novel cryptic binding sites that become exposed on u-PA and/or PAI-1 due to the complex formation. Recently Horn *et al.*[27] showed the presence of a cryptic binding site (Lysine 69) in the heparin binding domain of

PAI-1 that only interacts with LRP if PAI-1 was complexed with t-PA. It was also shown that Lysine 80 and 88, that are necessary for the binding of PAI-1 to heparin, contribute but little to the binding of LRP.

Since we determined that urinary tcu-PA is not recognized by LRP but by the asialoglycoprotein receptor (ASGPr) and heparan sulfate proteoglycans (HSPGs) on the liver[4], we were interested whether complex formation of urinary tcu-PA with PAI-1 resulted in the introduction of recognition of the complex by LRP.

Complex formation of tcu-PA with PAI-1 resulted in a 2-fold increased liver uptake at 10 min after injection and concurrently in a 1.6-fold increased serum clearance. Preinjection of GST-RAP reduced the maximum liver uptake of tcu-PA:PAI-1 2.2-fold, which indicates that LRP is responsible for the observed increased liver uptake. The maximal liver uptake of tcu-PA:PAI-1 after preinjection of GST-RAP is even 10 % lower than the liver uptake of uncomplexed tcu-PA (36.7 ± 1.7 % i.d. and 40.8 ± 1.6 % i.d. for tcu-PA:PAI-1 + GST-RAP and tcu-PA, respectively). At the same time, inhibition of the interaction of tcu-PA:PAI-1 with LRP delayed the serum clearance 2.3-fold. The contribution of the ASGPr to the liver uptake of the tcu-PA:PAI-1 complex was shown by the 1.2-fold inhibition of the liver uptake of tcu-PA:PAI-1 by the ASGPr antagonist ASOR.

Recently we showed that tcu-PA is besides by the ASGPr also recognized by HSPGs.[4] The contribution of the HSPGs in the interaction of tcu-PA:PAI-1 to the liver was determined by the use of the antagonist protamine sulfate since Narita *et al.*[32] showed that protamine sulfate specifically inhibited the binding of Tissue Factor Pathway Inhibitor to mouse liver HSPGs *in vivo*. Preinjection of protamine sulfate reduced the maximum liver uptake to 57.4 ± 1.4 % i.d. Concurrently the serum clearance was delayed 1.5-fold to 1.7 ± 0.2 ml/min. The relative contribution of LRP, ASGPr and HSPGs to the liver recognition of tcu-PA:PAI-1 was 54 % (LRP), 18 % (ASGPr) and 27 % (HSPGs). In comparison to uncomplexed tcu-PA the relative contribution of the ASGPr is reduced (42 % of the liver uptake of tcu-PA is mediated via the ASGPr, 18 % for tcu-PA:PAI-1 (table II)).[4] The contribution of the HSPGs in the liver uptake is increased upon complex formation from 204 fmol for tcu-PA to 827 fmol for tcu-PA:PAI-1 (table II). This can be explained by the recognition of PAI-1 via its heparin binding domain to HSPGs[23,24] and/or by an additional recognition of tcu-PA by HSPGs that is induced by the complex formation of tcu-PA with PAI-1.

The recognition of tcu-PA:PAI-1 by LRP was mainly caused by the Lysine residues 69, 80 and 88 within the heparin binding domain of PAI-1. For t-PA:PAI-1 it was shown recently[28] that residue 69 was mainly responsible for the recognition of the complex by LRP and that Lysine 69 was a cryptic binding site for LRP that only interacted with LRP when PAI-1 was complexed with t-PA. Mutation of Lysine 69, 80 and 88 within PAI-1 reduced the liver uptake of tcu-PA:(K69A,K80A,K88A)-PAI-1 from 79.1 ± 2.4 % i.d. to 52.1 ± 3.2 % i.d.,

concurrently the serum clearance is delayed 1.7-fold. To further elucidate the role of Lysine 69 in the interaction with LRP, the *in vivo* behaviour of tcu-PA complexed with (K69A)-PAI-1 was studied. Tcu-PA:(K69A)-PAI-1 showed a maximum liver uptake and serum clearance value (liver uptake: 47.4 ± 3.4 % i.d., clearance: 1.6 ± 0.1 ml/min) comparable to that of tcu-PA:(K69A,K80A,K88A)-PAI-1. It can be concluded that Lysine 69 in the heparin binding domain of PAI-1 is the major residue responsible for the interaction of tcu-PA:PAI-1 with LRP and that residue 80 and 88 are not involved in the interaction of PAI-1 with LRP.

A preinjection of GST-RAP reduced the liver uptake of tcu-PA:(K69A,K80A,K88A)-PAI-1 and tcu-PA:(K69A)-PAI-1 to 29.6 ± 1.3 % i.d. and 30.4 ± 3.2 % i.d., respectively. Since the liver uptake of tcu-PA complexed with (K69A,K80A,K88A)-PAI-1 or (K69A)-PAI-1 with the preinjection of GST-RAP is reduced more than the liver uptake of tcu-PA which is not recognized by LRP[4] (40.8 ± 1.6 % i.d.), it may be concluded that due to the complex formation of tcu-PA with PAI-1 cryptic binding sites not only within PAI-1 (Lysine 69)[27] but also within tcu-PA are exposed that mediate the interaction with LRP.

The effect of mutation of residue 69, 80 and 88 in PAI-1 on the recognition by HSPGs is difficult to determine since the reduction of the interaction with LRP may be compensated by an increased recognition of the mutant PAI-1 complexes by HSPGs and/or other proteoglycans in the liver and organs in the body and deletion of some residues in the heparin binding domain of PAI-1 may change the ability of this domain to interact with proteoglycans. Further investigation is needed with specific (unavailable) inhibitors for various proteoglycans to exactly elucidate the contribution of proteoglycans to the recognition of tcu-PA:PAI-1 *in vivo*.

Comparable results as for tcu-PA:PAI-1 were obtained for t-PA complexed with PAI-1, (K69A,K80A,K88A)-PAI-1 or (K69A)-PAI-1. Complex formation of t-PA with PAI-1 resulted in a 1.2-fold induced liver uptake to 94 % i.d. Mutation of the lysine 69 that is mainly responsible for the interaction of PAI-1 with LRP resulted by analogy to tcu-PA in the reduction of the liver uptake to that of uncomplexed t-PA (77 % i.d.). No additional effect was observed when besides lysine 69, also lysine 80 and 88 were mutated. Therefore, we may conclude that the cryptic lysine 69 binding site in the heparin binding domain of PAI-1 is the determining residue for the recognition of PAI-1 complexed to plasminogen activators.

In conclusion, this study shows that complex formation of urinary tcu-PA with its physiological inhibitor PAI-1 results in the initiation of recognition of the tcu-PA:PAI-1 complex by LRP. Due to the complex formation cryptic LRP binding sites in PAI-1 (lysine 69) and probably in tcu-PA are exposed that are responsible for the recognition of tcu-PA:PAI-1 by LRP.

ACKNOWLEDGEMENT

This study was supported by the Netherlands Heart Foundation (grant 92.139).

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Chapter 8

Blockade of the α_2-Macroglobulin Receptor/Low Density Lipoprotein Receptor-related Protein on rat liver parenchymal cells by the 39 kDa Receptor-Associated Protein leaves the interaction of ß-migrating Very-Low Density Lipoprotein with the lipoprotein remnant receptor unaffected

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SUMMARY

The nature of the liver binding site which is responsible for the initial recognition and clearance of chylomicron-remnants and ß-migrating Very-Low Density Lipoprotein (ß-VLDL) is under active dispute. We have investigated the effect of the 39 kDa Receptor-Associated Protein (RAP) on the recognition site for activated α_2-macroglobulin and ß-VLDL on rat parenchymal liver cells *in vivo* and *in vitro* in order to analyze whether both substrates are recognized and internalized by the same receptor system. Radiolabelled trypsin-activated α_2-macroglobulin (α_2-M-T) was cleared rapidly by the liver (maximal uptake of 80.8 ± 1.0 % of the injected dose). Preinjection of 5, 15, or 50 mg gluthathione-S-transferase-linked RAP (GST-RAP)/kg rat reduced the liver uptake to 65.0 ± 0.7 %, 58.1 ± 0.8 % or 2.9 ± 0.1 % of the injected dose, respectively. Concurrently the serum decay was strongly delayed after preinjection of 50 mg GST-RAP/kg rat but this did not affect the serum decay and liver uptake of ^{125}I-ß-VLDL. Binding studies with isolated parenchymal liver cells *in vitro* demonstrated that the binding of ^{125}I-α_2-M-T was inhibited for 98 % by GST-RAP with an IC_{50} of 0.3 μg/ml (4.2 nM), whereas the binding of ^{125}I-ß-VLDL and ^{125}I-ß-VLDL+recombinant apolipoproteinE (rec-apoE) was unaffected by GST-RAP upto 50 μg/ml (700 nM). Also, the cell-association and degradation of α_2-M-T was blocked by GST-RAP, while the association and degradation of ß-VLDL and ß-VLDL +rec-apoE were not influenced. The inhibitory effect of GST-RAP on the cell-association and degradation of α_2-M-T lasted for 1-2 h of incubation at 37 °C. The binding of the radioiodinated GST-RAP to isolated parenchymal liver cells was highly efficiently coupled to lysosomal degradation. Upon *in vivo* injection into rats, ^{125}I-labelled GST-RAP is rapidly cleared from the serum and taken up by the liver, which is also coupled to efficient degradation. Since GST-RAP blocks the binding of all known ligands to the α_2-Macroglobulin Receptor/Low Density Lipoprotein Receptor-related Protein (the α_2-MR/LRP) and at high concentrations the binding to the LDL receptor, we conclude that the initial binding and internalization of ß-VLDL by rat parenchymal liver cells is not mediated by the α_2-MR/LRP. The properties of binding of ß-VLDL to rat parenchymal liver cells points to an apoE-specific recognition site for lipoprotein remnants which differs from the α_2-MR/LRP, proteoglycans and the LDL receptor and is tentatively called the lipoprotein remnant receptor.

INTRODUCTION

Chylomicrons and Very-Low Density Lipoprotein (VLDL) interact with lipoprotein lipase after entering the blood circulation. This interaction leads to

hydrolysis of most of their triacylglycerols.[1] During this process, the apolipoprotein (apo) pattern of the formed lipoprotein remnants shows a relative increase in apoE.[2] Subsequently apoE functions as a recognition marker for receptor-mediated uptake by the parenchymal liver cells.[2-4] The interaction of chylomicron-remnants and ß-VLDL with parenchymal liver cells can be blocked by lactoferrin[4,5], an Fe^{3+}-carrying protein with an Arg+Lys-rich sequence at positions 25-31 which resembles the binding site of apoE (amino acids 142-148).[6]

The nature of the recognition site on parenchymal liver cells that recognizes apoE is under discussion.[4,7-12] Choi and Cooper[7] found that an antibody to the Low Density Lipoprotein (LDL) receptor reduced the uptake of radioiodinated chylomicron-remnants by the liver to about 50 %. In contrast, Kita *et al.*[13] found no uptake of abnormal lipoprotein remnants in the Watanabe heritable hyperlipidaemic rabbit, an animal with dysfunctional LDL receptors. Similarly in humans, the lack of LDL receptors does not lead to a pathological change in the metabolism of dietary fat.[14]

Candidate proteins, which might function as an initial recognition site for remnants, are the lipolysis-stimulated receptor, proteoglycans, and the LDL Receptor-related Protein (LRP). The lipolysis-stimulated receptor is a receptor that is activated by free fatty acids and has a high affinity for triacylglycerol-rich lipoproteins.[12] Proteoglycans are proteins that have one or more attached glycosaminoglycan chains, with highly negatively charged sulfate and carboxylate groups. A large number of ligands are known to bind to proteoglycans, including apoE[15], apoE-enriched lipoproteins[11] and lactoferrin.[16-18]

The identification by Herz *et al.*[19] of a 600 kDa protein with structural similarity to the LDL receptor, called LRP, has led to the suggestion that this protein can function as lipoprotein remnant receptor.[20] However, LRP only recognizes lipoprotein remnants which are enriched with apoE *in vitro*.[21,22] The debate whether the lipoprotein remnant receptor is LRP has been intensified by the finding that the α_2-macroglobulin receptor (α_2-MR) and LRP are the same molecule.[23,24] It is generally accepted that the α_2-MR/LRP is a multifunctional receptor as it recognizes, in addition to activated α_2-M and apoE-enriched ß-VLDL, complexes between recombinant tissue-type plasminogen activator and plasminogen activator inhibitor type-1[25,26], complexes between urokinase-type plasminogen activator and plasminogen activator inhibitor type-1[27,28], *Pseudomonas* exotoxin A[29], chicken vitellogenin[30], lipoprotein lipase[31], and bovine lactoferrin.[32]

A 39 kDa Receptor-Associated Protein (RAP), as identified by Strickland and coworkers[23] and Kristensen *et al.*[24] can bind with high affinity to the LRP, thereby blocking the binding of all known ligands to the receptor.[33] RAP functions intracellularly as a molecular chaperon for LRP and maintains LRP in an inactive ligand-binding state.[34] Recently Willnow *et al.*[35] have shown that RAP-deficient mice show a significant reduction in LRP expression, resulting in an impaired clearance of methylamine activated α_2-M by the liver.

In the present studies we used RAP to study the nature of the ß-VLDL recognition site on parenchymal liver cells and its relation with the α_2-MR/LRP. The effect of RAP on the recognition sites of activated α_2-M and ß-VLDL have been compared directly *in vivo* and *in vitro* in order to analyze whether the initial recognition sites share common properties or that additional recognition systems for ß-VLDL do exist on parenchymal liver cells.

MATERIALS AND METHODS

Chemicals

Trypsin (EC 3.4.21.4) from bovine pancreas and soybean trypsin inhibitor were from Boehringer Mannheim (Germany). BSA (fraction V) and collagenase type IV (clostridiopeptidase A, EC 3.4.24.3) were from Sigma (U.S.A.). ^{125}I (carrier free) in NaOH was from Amersham (U.K.). Bio-Gel A-1.5m was from Bio-Rad (U.S.A.). Dulbecco's modified Eagle's medium (DMEM) was from Gibco (U.K.). Recombinant human apoE (rec-apoE) was a generous gift from Tikva Vogel, Bio-Technology General, Ltd. (Israel), and was supplied as a lyophilized powder containing 76 % rec-apoE, 11.7 % L-cysteine, and 12 % $NaHCO_3$.[36] All other chemicals were of analytical grade.

Animals

For isolation of ß-VLDL, six to eight rats, with a mass of 200-220 g each, were maintained for 16 days on a cholesterol-rich chow (Hope Farms, Woerden, The Netherlands) that included 2 % (w/w) cholesterol, 5 % (w/w) olive oil, and 0.5 % (w/w) cholic acid.

ß-VLDL, α_2-macroglobulin and GST-RAP

ß-VLDL was obtained from cholesterol-fed rats that were starved for 20 h, after which blood was collected by puncture of the abdominal aorta. The sera were pooled and ß-VLDL was isolated as described by Harkes *et al.*[37] Composition of ß-VLDL was 14.6 ± 2.1 % triglycerides, 15.8 ± 1.1 % phospholipids, 49.4 ± 3.1 % esterified cholesterol, 9.9 ± 1.0 % free cholesterol, and 10.3 ± 0.7 % protein. Enrichment of ß-VLDL with rec-apoE was performed as previously described by Kowal *et al.*[22] Human α_2-macroglobulin (α_2-M) was isolated as described previously[4], and activated with trypsin by incubation with a 15-fold molar excess of trypsin for 5 min at 20 °C followed by a 5-fold molar excess of soybean trypsin inhibitor as related to trypsin.[38] In a subsequent step, trypsin-activated α_2-M (α_2-M-T) was separated from smaller protein complexes by gel filtration on a 0.7 x 25-cm Bio-Gel A-1.5m column with phosphate buffered saline (10 mM phosphate buffer containing 150 mM NaCl and 1 mM EDTA, pH 7.4; NaCl/Pi/EDTA) as the eluent.

A plasmid (pGEX) encoding for a fusion protein (GST-RAP) of glutathione S-transferase (GST) and the 39 kDa protein or Receptor Associated Protein (RAP), which was transformed in *E.coli* (DH5α), was a generous gift of Dr. J. Herz (Dallas, Texas, USA). GST-RAP was produced as described by Herz *et al.*[39] Transformed *E.coli* were cultured at 37 °C to an OD_{600} of 0.4-0.5 and transcription of GST-RAP was induced with isopropylthio-ß-D-galactoside (0.01%) and growth was continued for 6 hours at 37°C. Cells were harvested by centrifugation at 4° C and GST-RAP was isolated from the solubilized cells using GSH-sepharose. The protein content of the isolated GST-RAP was determined by the method of Lowry[40] with BSA as standard. The GST-RAP solution was subsequently concentrated to a concentration of 10 mg of GST-RAP/ml using polyethyleenglycol 8000. The isolated GST-RAP was for more than 95 % pure as determined by analysis on 10 % SDS-PAGE. The activity of GST-RAP was tested by determining the ability of GST-RAP to inhibit the binding of ^{125}I-α_2-M-T to freshly isolated rat parenchymal liver cells. The displacement of the binding of ^{125}I-α_2-M-T (3.6 nM) by GST-RAP was 4.2 nM, which is similar to the IC_{50} observed for GST-RAP kindly donated by Dr. D.K. Strickland (Rockville, Maryland, USA). The purified GST-

RAP was dialyzed against NaCl/Pi before administration to rats. In some experiments RAP was cleaved from the GST-RAP fusion protein by incubation with thrombin, essentially as described by Williams *et al.*[41]

Labelling of ß-VLDL, α_2-M and GST-RAP

ß-VLDL was radioiodinated at pH 10 with carrier-free ^{125}I according to a modification[42] of the ICl method.[43] Free ^{125}I was removed by Sephadex G-25 gel filtration with NaCl/Pi/EDTA as the eluent followed by dialysis against NaCl/Pi/EDTA, pH 7.4, for 20 h at 4 °C with repeated changes of buffer. The distribution of radioactivity in ^{125}I-*ß*-VLDL was 85.5 ± 3.2 % in protein, 12.6 ± 3.6 % in lipid, and 1.9 ± 0.8 % unbound.

Unactivated α_2-M was radioiodinated with chloramine T as published previously.[38] In brief, 350 μg of α_2-M in 200 μl of NaCl/Pi, pH 8.0, was mixed with 8 μl of ^{125}I in 0.1 M NaOH followed by 214 μg of chloramine T in 107 μl of NaCl/Pi, pH 8.0. After 90 s, the reaction was stopped by the addition of 164 μg of $Na_2S_2O_5$ in 82 μl of NaCl/Pi, pH 8.0, and 120 μg of KI in 60 μl of NaCl/Pi, pH 8.0. In a subsequent step, ^{125}I-α_2-M was directly activated with trypsin by incubation with a 15-fold molar excess of trypsin for 5 min at 20 °C followed by a 5-fold molar excess of soybean trypsin inhibitor as related to trypsin.[38] Isolation of ^{125}I-α_2-M-T was similar to the isolation of α_2-M-T described above. The specific activities of ^{125}I-α_2-M-T preparations ranged from 480 to 1140 dpm/ng of protein. ^{125}I-α_2-M-T was stored at -80 °C for no longer than 14 days.

Recombinant GST-RAP and the RAP were radioiodinated by the Iodogen method.[44] Labelling of GST-RAP with ^{125}I-tyramine-cellobiose (^{125}I-TC) was performed as described by Pittman *et al.*[45]

Serum decay and liver uptake

Male Wistar rats with a mass of 225-300 g, fed with regular chow, were used in this study. Rats were anaesthetized by intraperitoneal injection of 15-20 mg of sodium pentobarbital. Radiolabelled ligands were injected via the vena cava inferior. At the indicated times, blood samples of 0.3 ml were taken from the vena cava inferior and allowed to clot for 30 min. The samples were centrifuged for 2 min at 16,000 g, and 100-μl serum samples were counted. The total amount of radioactivity in the serum was calculated as follows: serum volume (ml) = (0.0219 x body weight (g)) + 2.66.[46]

At the indicated times, liver lobules were excised and weighed, and radioactivity was counted. At the end of the experiment, the remainder of the liver was excised and weighed. The amount of liver tissue tied off at the end of the experiment did not exceed 15% of the total liver weight. Radioactivity was corrected for the radioactivity in serum assumed to be present in the liver at the time of sampling (85 μl/g, wet weight).[47]

Studies with freshly isolated parenchymal liver cells *in vitro*

Parenchymal liver cells were isolated by perfusion of the liver with 0.05% collagenase by the method of Seglen[48] modified as described previously.[49] The obtained parenchymal liver cells were resuspended in DMEM containing 2 % BSA, pH 7.4. For inhibition experiments, 1×10^6 parenchymal cells (> 95 % viable, as judged by 0.2 % Trypan Blue exclusion) were incubated with 5 μg/ml ^{125}I-*ß*-VLDL or 2.6 μg/ml ^{125}I-α_2-M-T, and the indicated amounts of competitor in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 % BSA, pH 7.4. Incubations were carried out in plastic tubes (Kartell) for 2 h at 4 °C, in a circulating lab shaker at 150 rpm. For incubations at 37 °C, the air in the tube was saturated with carbogen (95 % O_2, 5% CO_2) every 30 min. About 1×10^6 parenchymal cells (> 95% viable) were incubated with 2.6 μg/ml of ^{125}I-α_2-M-T or 5 μg/ml of ^{125}I-*ß*-VLDL in the presence or absence of 10 μg/ml of GST-RAP, or with ^{125}I-GST-RAP in the presence or absence of 100 μM of chloroquine, for the indicated period of time at 37 °C in DMEM, pH 7.4, containing 2 % BSA, under continuous shaking. After incubation, the cells were centrifuged at 50 xg for 1 min at 4 °C and washed twice with washing buffer (0.9 % NaCl, 1 mM EDTA, 0.05 M Tris-HCl, 5 mM $CaCl_2$, 0.2 % BSA, pH 7.4) and once with washing buffer without BSA. Cells were lysed in 1 ml of 0.1 M NaOH, and subsequently radioactivity and protein content

were determined. Degradation of the ligands was determined as follows: to 0.5 ml of the first supernatant, 0.2 ml of 35 % trichloroacetic acid was added followed by incubation at 37 °C for 30 min; the mixture was then centrifuged for 2 min at 15,900 xg. To 0.5 ml of the supernatant obtained after precipitation of the first supernatant with trichloroacetic acid, 10 μl of 20 % KI and 25 μl of 30 % H_2O_2 were added. After 5 min at room temperature, 0.8 ml of $CHCl_3$ was added and the mixture was shaken for 15 min. After centrifugation for 2 min at 15,900 xg the aqueous phase was counted. Protein contents were determined according to Lowry *et al.*[40] with BSA as internal standard.

RESULTS

Effect of GST-RAP on the serum decay and liver uptake of activated ^{125}I-α_2-M-T and ^{125}I-ß-VLDL *in vivo*

Upon injection of α_2-M-T into rats a rapid clearance is observed. The liver is mainly responsible for the uptake.[50] The liver uptake reached its maximum at 10 min after injection (80.8 ± 1.0 % of the injected dose; figure 1A). Figure 1B shows that α_2-M-T was cleared with a half-life of 1.3 ± 0.1 min. Preinjection of 5, 15, or 50 mg of GST-RAP/kg rat 1 minute prior to the injection of ^{125}I-α_2-M-T reduced the maximal liver uptake at 10 min after injection from 80.8 ± 1.0 % to 65.0 ± 0.7, 58.1 ± 0.8, or 2.9 ± 0.1 % of the injected dose, respectively (figure 1A). Concurrently the serum half-life of ^{125}I-α_2-M-T was lengthened from 1.3 ± 0.1 to 76.2 ± 0.8 min.

Radiolabelled ß-VLDL is cleared rapidly from the circulation (half-life of 0.8 ± 0.1 min) by the liver (maximal uptake of 61.5 ± 2.4 % of the injected dose at 10 min after injection). Injection of 50 mg GST-RAP/kg rat prior to the injection of ^{125}I-ß-VLDL did not affect the liver uptake (maximal uptake 60.3 ± 2.7 % of the injected dose at 10 min after injection; figure 1C) and serum clearance (half-life of 0.8 ± 0.2 min; figure 1D).

Effect of GST-RAP on the binding of ^{125}I-α_2-M-T, ^{125}I-ß-VLDL, and ^{125}I-ß-VLDL+rec-apoE to rat parenchymal liver cells

Isolated rat parenchymal liver cells bind α_2-M-T with high affinity.[38,51] In figure 2 it is shown that GST-RAP is a very effective inhibitor of the binding of ^{125}I-α_2-M-T to the parenchymal liver cells. An almost complete (98 %) inhibition of the binding of ^{125}I-α_2-M-T by GST-RAP with an IC_{50} of 0.3 μg/ml (4.2 nM) was observed (figure 2A).

Isolated parenchymal liver cells bind rat ß-VLDL in a saturable way by which an efficient cross-competition with chylomicron-remnants is observed whereas high levels of LDL are ineffective.[4] GST-RAP, up to a concentration of 50 μg/ml, did not affect the binding of ^{125}I-ß-VLDL or ^{125}I-ß-VLDL+rec-apoE (figure 2B) whereas GST-RAP is extremely effective in blocking the ^{125}I-α_2-M-T binding.

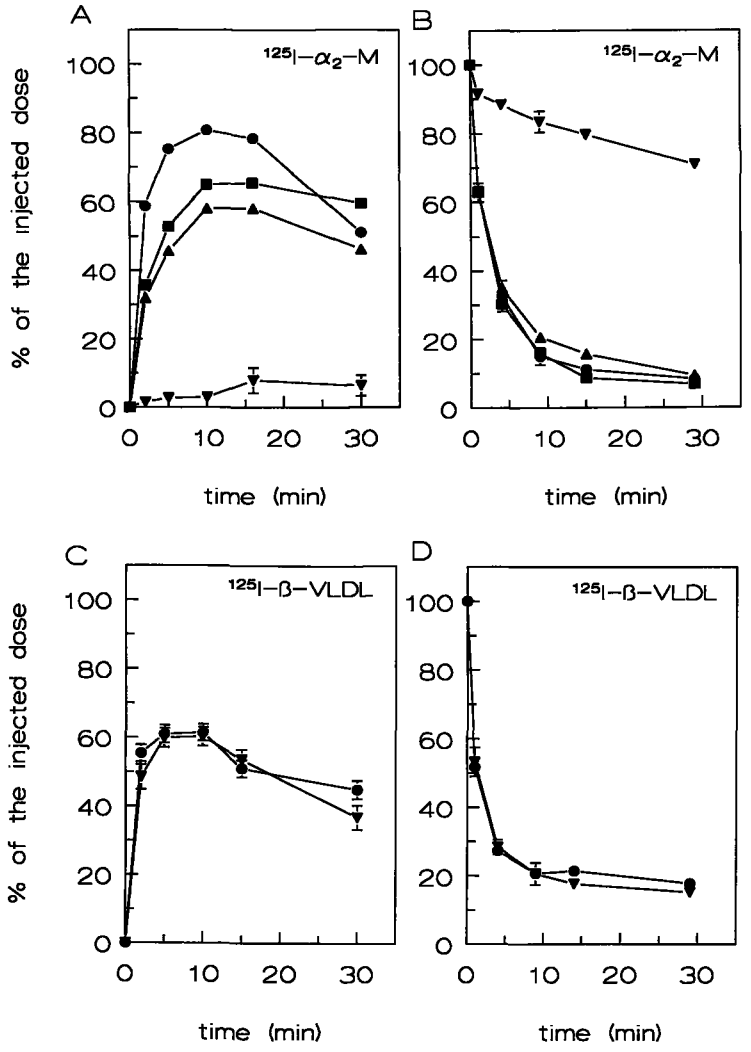

Figure 1. Effect of GST-RAP on the liver uptake and serum decay of ^{125}I-α_2-M-T or ^{125}I-ß-VLDL in the rat *in vivo*. Rats were injected intravenously with 0 (●), 5 (■), 15 (▲), or 50 (▼) mg GST-RAP/kg body weight 1 min prior to injection of 8 μg/kg body weight ^{125}I-α_2-M-T (A, B) or 50 μg/kg body weight ^{125}I-ß-VLDL (C, D). Radioactivity in liver (A, C) and serum (B, D) were determined at the indicated times. Results are expressed as the percentage of the injected dose ± S.E.M. ($n = 3$). When not visible error bars are within the symbol size.

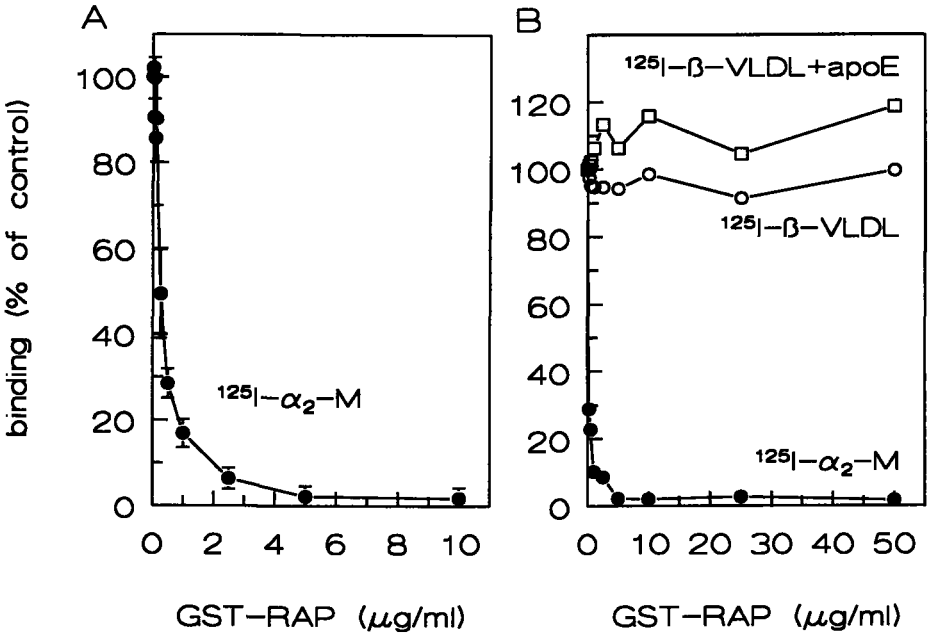

Figure 2. Effect of GST-RAP on ^{125}I-α_2-M-T, ^{125}I-ß-VLDL and ^{125}I-ß-VLDL+rec-apoE binding to rat parenchymal liver cells. Freshly isolated parenchymal liver cells were incubated for 2 h at 4 °C with 2.6 μg/ml ^{125}I-α_2-M-T (●), 5 μg/ml ^{125}I-ß-VLDL (O) or 5 μg/ml ^{125}I-ß-VLDL+rec-apoE (□) and the indicated concentrations of GST-RAP. Binding is expressed as percentage of the binding of 2.6 μg/ml ^{125}I-α_2-M-T or 5 μg/ml ^{125}I-ß-VLDL (+rec-apoE) in the absence of GST-RAP. Results are given as means ± S.D. (n = 3). When not visible, error bars are within the symbol size.

Effect of GST-RAP on α_2-M-T and ß-VLDL handling

The α_2-MR/LRP has been implicated in the cellular uptake of ligands which are initially bound to other sites on the cell-surface, i.e. extracellular proteoglycans.[11,33] In order to test whether the degradation of α_2-M-T and ß-VLDL was modified by GST-RAP, we incubated the parenchymal cells at 37 °C and determined the effect of GST-RAP on the cell-association and degradation of ^{125}I-α_2-M-T (figure 3A) and ^{125}I-ß-VLDL (figure 3B). In the absence of GST-RAP, ^{125}I-α_2-M-T became rapidly associated to the cells, whereas after 60 min of incubation, an apparent equilibrium value was reached (figure 3A). The degradation of ^{125}I-α_2-

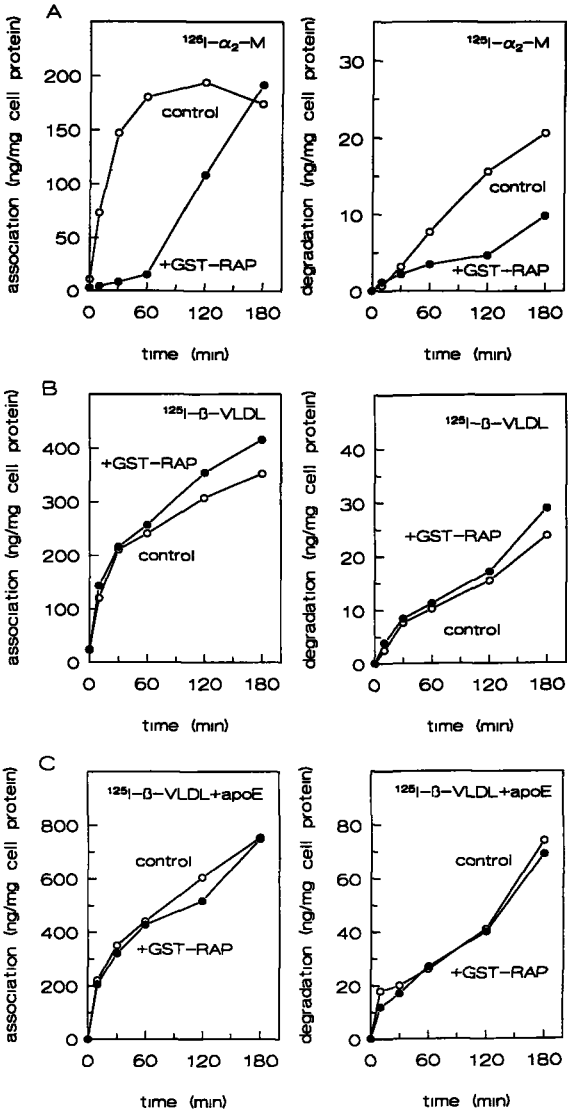

Figure 3. Effect of GST-RAP on the cell association and degradation of ^{125}I-α_2-M-T, ^{125}I-ß-VLDL and ^{125}I-ß-VLDL+rec-apoE by parenchymal liver cells. Freshly isolated parenchymal liver cells were incubated for the indicated period of time at 37 °C with 2.6 µg/ml ^{125}I-α_2-M-T (A), 5 µg/ml ^{125}I-ß-VLDL (B) or 5 µg/ml ^{125}I-ß-VLDL+rec apoE (C) in the absence (O) or presence (●) of 10 µg/ml of GST-RAP. Cell association and degradation were determined and are expressed as ng ^{125}I-α_2-M-T or ^{125}I-ß-VLDL/mg cell protein. Results are given as means ± S.D. (n = 3). When not visible, error bars are within the symbol size.

M-T followed a different time-dependency by which, after an initial lag-phase of about 10 min, a constant increase in degradation products was observed (figure 3A). In the presence of 10 μg/ml GST-RAP, the initial cell-association was almost completely blocked, but surprisingly at 120 min of incubation the effect of GST-RAP diminished and at 180 min a similar value as in the control incubation was found. GST-RAP also transiently suppressed the degradation of ^{125}I-α_2-M-T upto 120 min of incubation whereas between 120 and 180 min of incubation the increase in trichloroacetic-acid-soluble degradation products was similar to the control incubation (figure 3A). When ^{125}I-β-VLDL or ^{125}I-β-VLDL+rec-apoE were incubated with the parenchymal cells, no effect of GST-RAP on the cell-association and degradation was noticed (figure 3B, C).

Binding and handling of GST-RAP by the parenchymal liver cells

The finding that, upon incubation of ^{125}I-α_2-M-T with parenchymal cells at 37 °C, only a transient inhibition by GST-RAP of the cell-association and degradation was noticed, suggested to us that GST-RAP might be internalized and degraded by the cells. It appeared that unlabelled GST-RAP very efficiently competes for the binding of ^{125}I-GST-RAP (figure 4). The glutathione S-transferase appears not to be responsible for this high affinity interaction because RAP from which the GST moiety has been removed is similarly effective in the competition assay (data not shown).

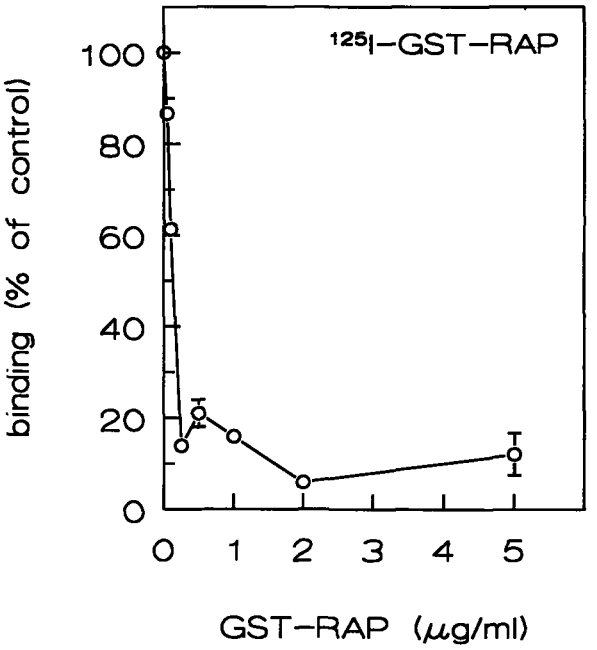

Figure 4. Effect of unlabelled GST-RAP on the binding of ^{125}I-GST-RAP to parenchymal liver cells. Freshly isolated liver cells were incubated with 0.5 μg/ml of ^{125}I-GST-RAP in the presence of the indicated concentrations of GST-RAP (O). Binding is expressed as percentage of the binding of ^{125}I-GST-RAP in the absence of competitor. Results are given as means ± S.D. (n = 3).

Upon incubation of ^{125}I-GST-RAP with parenchymal cells at 37 °C, an increased cell-association occurred with time, which reached an apparent equilibrium at 120 min of incubation (figure 5). Degradation of the ^{125}I-GST-RAP was observed after an initial lag phase of 10 min (figure 5). Chloroquine, a lysosomotropic agent which blocks lysosomal degradation, greatly inhibits the degradation of ^{125}I-GST-RAP, suggesting that degradation occurs in the lysosomes (figure 5). It is clear however, that an inhibition of the degradation is not coupled to an increased accumulation of radioactive ligand in the cells because the cell-association is also lowered by the presence of chloroquine, especially at the later incubation times.

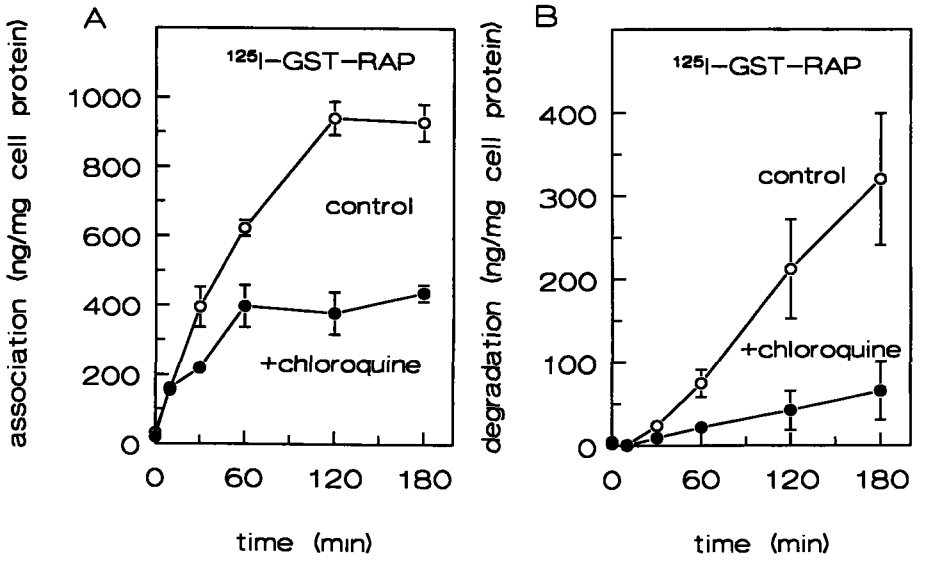

Figure 5. Effect of chloroquine on the cell association and degradation of ^{125}I-GST-RAP by parenchymal liver cells. Freshly isolated parenchymal liver cells were incubated with 10 μg/ml of ^{125}I-GST-RAP in the presence (●) or absence (○) of 100 μM chloroquine. Cell association (A) and degradation (B) were determined and are expressed as ng ^{125}I-GST-RAP/mg cell protein. Results shown are means ± S.D. (n = 3). When error bars are not visible, errors are within the symbol size.

In vivo fate of GST-RAP

Upon injection of ^{125}I-GST-RAP into rats, a rapid decay from the blood circulation was noticed, coupled to a quantitative uptake in the liver (figure 6). Between 10 to 30 min after injection the liver-associated radioactivity declined, whereas the serum radioactivity remained constant. In order to investigate whether the decline in liver radioactivity was caused by degradation of ^{125}I-GST-RAP we labelled the protein with ^{125}I-tyramine-cellobiose, a radiolabel which remains associated with cells after degradation of the labelled substrate.[45] No decline in liver radioactivity occurred (figure 6), indicating that the decline in radioactivity between 10-30 min after injection of ^{125}I-GST-RAP in the liver was not caused by resecretion of intact protein, but was caused by degradation.

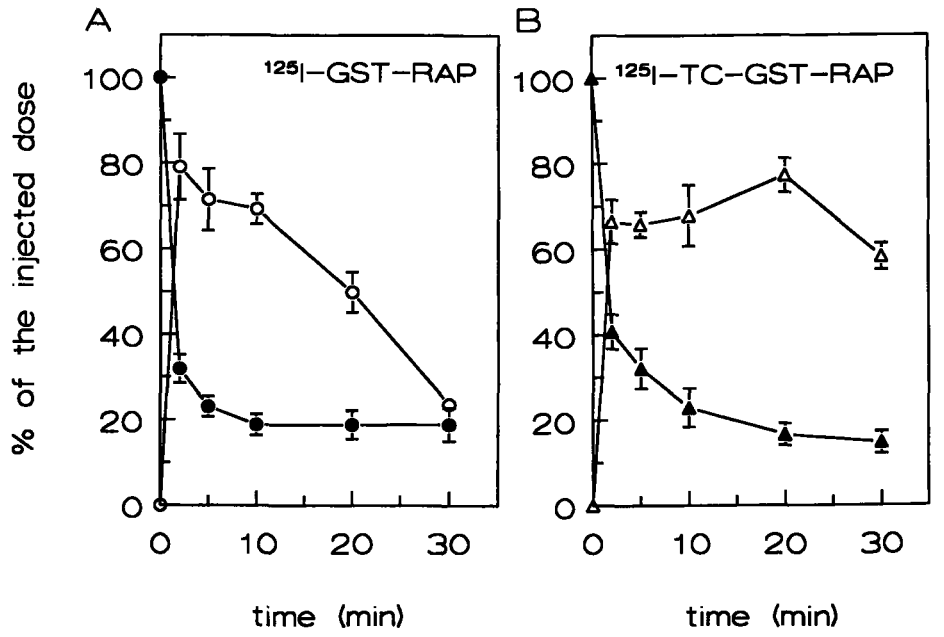

Figure 6. Serum decay and liver uptake of ^{125}I- or ^{125}I-tyramine-cellobiose-labelled GST-RAP in the rat *in vivo*. ^{125}I-labelled GST-RAP (A) or ^{125}I-tyramine-cellobiose-labelled GST-RAP (B) was injected intravenously (5 μg/kg body weight) into anaesthetized rats. Radioactivity in serum (●, ▲) and liver (O,Δ) was determined at the indicated times. Results are expressed as the percentage of the injected dose. Results shown are means ± S.D. (n = 3). When error bars are not visible, errors are within the symbol size.

DISCUSSION

The α_2-Macroglobulin Receptor/Low Density Lipoprotein Receptor-related Protein (α_2-MR/LRP) is a member of the LDL-receptor gene family, which comprises seven known cell-surface receptors, namely (1) the LDL receptor, (2) the VLDL receptor[52], (3) the vitellogenin receptor[53], (4) the α_2-MR/LRP[19], (5) an α_2-MR/LRP-like protein from the nematode *Caenorhabditis elegans*[54], (6) gp330[55], and (7) a G protein-coupled receptor with LDL-binding motifs from the mollusc *Lymnaea stagnalis*.[56]

The α_2-MR/LRP and gp330, in particular, share substrate recognition and can be considered multifunctional. Among the established substrates for α_2-MR/LRP the recognition of activated α_2-M is without dispute.[33] In the present paper we used α_2-M activated by trypsin (α_2-M-T) as a reference substrate in order to analyse the suggested role of the α_2-MR/LRP as initial recognition site for ß-VLDL and chylomicron-remnants.[20] Furthermore we used a 39kDa Receptor-Associated Protein (RAP) that binds with high affinity to the α_2-MR/LRP and blocks effectively the binding of α_2-M-T.[39,41]

Preinjection of 50 mg GST-RAP/kg rat significantly reduced the liver uptake of α_2-M-T (from 80.8 ± 1.0 % to 2.9 ± 0.1 % of the injected dose). Concurrently the serum half-life was lengthened from 1.3 ± 0.1 min to 76.2 ± 0.8 min. The serum decay and liver uptake of ^{125}I-ß-VLDL were not affected by a preinjection of 50 mg GST-RAP/kg rat. Willnow *et al.*[57] showed that adenovirus containing the RAP cDNA, upon intravenous injection can lead to circulating levels of the RAP of 400 µg/ml. In wild type mice no hyperlipidemia is induced, while total cholesterol is increased 4-fold in LDL-receptor negative mice at 5 days after virus injection.[57] α_2-M clearance was reduced in these mice while no data were presented on the clearance of lipoprotein remnants. Our results are in agreement with the suggestion of Willnow *et al.*[57] that the first sequestration of remnants in the liver may reside on molecules different from the α_2-MR/LRP.

We observed with isolated rat parenchymal liver cells that GST-RAP inhibits the binding of ^{125}I-α_2-M-T completely with an IC_{50} of 4.2 nM, whereas the binding of ^{125}I-ß-VLDL and ^{125}I-ß-VLDL+rec-apoE to the cells was unaffected upto 700 nM of GST-RAP. RAP blocks binding of all known ligands to the α_2-MR/LRP[33], very high concentrations of RAP (> 500 nM) also efficiently block the LDL receptor-mediated binding.[58] We therefore conclude that the initial binding of ß-VLDL to rat liver parenchymal liver cells is not mediated by the α_2-MR/LRP or the LDL receptor. For the complex between urokinase-type plasminogen activator and plasminogen activator inhibitor type-1 it has been proposed that initial binding of this complex occurs to the urokinase-type plasminogen activator receptor, a step which precedes internalization by the α_2-MR/LRP.[28,59] A similar mechanism might be operative for apoE and lipoprotein lipase.[33] In order to test the possibility of

such a mechanism for ß-VLDL, we also analysed the effect of GST-RAP at 37 °C on the cell-association and degradation of ß-VLDL and α_2-M-T. Both cell-association and degradation of ^{125}I-α_2-M-T were greatly blocked by the GST-RAP, whereas the cell-association and degradation of ^{125}I-ß-VLDL were unaffected. From these data it appears that also the internalization and degradation of ß-VLDL by rat parenchymal liver cells is not influenced by RAP, suggesting that a receptor, different from the α_2-MR/LRP, is responsible both for recognition and intracellular handling.

A surprising finding was that the effect of GST-RAP on the cell-association and degradation of ^{125}I-α_2-M-T lasted only for 1 to 2 h. Preincubations of GST-RAP at 37 °C did not affect its capacity to inhibit the binding of ^{125}I-α_2-M-T, indicating that the temporarily inhibition is not caused by an intrinsic instability of the inhibitor at 37 °C (results not shown). It appears that GST-RAP is a high-affinity substrate for rat parenchymal liver cells and its binding is efficiently coupled to degradation. The degradation of GST-RAP is inhibited by chloroquine, suggesting that it occurs in the lysosomes. In addition to an effective inhibition of the degradation, chloroquine also inhibited the cell-association especially at later incubation times. This effect was specific for GST-RAP as the association of ^{125}I-α_2-M-T was not affected by the addition of chloroquine (data not shown). It might be possible that in the presence of chloroquine, GST-RAP does not dissociate from α_2-MR/LRP, leading to intracellular entrapment of the receptor, or that chloroquine does influence the (re)recruitment of the receptor on the cell-membrane. Although the effective degradation of GST-RAP by the rat parenchymal liver cells may explain the transient inhibition, we calculated that additional (extracellular) inactivation must occur in order to explain the lack of inhibition after 1 h of incubation. Further experiments will be needed in order to analyse the receptors involved in GST-RAP binding to rat parenchymal liver cells as it was recently reported that, in addition of binding to α_2-MR/LRP, RAP also binds to the LDL receptor[58-60] and gp330.[61,62] It is however clear that also *in vivo* RAP is very effectively recognized and degraded by the liver. These data are consistent with results obtained by Warshawsky *et al.*[63] who also showed that the liver was mainly responsible for the uptake of RAP while Iadonato *et al.*[64] described rapid uptake and degradation of RAP by rat hepatoma cells.

There is no doubt that the α_2-MR/LRP is able to interact with ß-VLDL enriched in apoE *in vitro*.[20-22,65] However, as shown earlier[4] with isolated parenchymal liver cells, ß-VLDL and chylomicron-remnants do cross-compete for a high-affinity binding site (tentatively called lipoprotein remnant receptor) whereas high concentrations of LDL are ineffective. Choi and Cooper[7] described how antibodies against the LDL-receptor reduced remnant uptake in the liver by 40 % while GST-RAP had little effect on the initial uptake of chylomicron-remnants by the liver. However, GST-RAP does block effectively the interaction of LDL with the LDL receptor in estradiol-treated rats[60] while Medh *et al.*[58] showed recently that

high concentrations of RAP also block the interaction of VLDL and LDL with the LDL-receptor of fibroblasts. The ineffectivity of GST-RAP to affect the ß-VLDL interaction with rat hepatocytes, while the interaction of α_2-M-T is very efficiently and completely blocked, thus confirms that the LDL-receptor is not responsible for the primary interaction. This is also consistent with the data of Kita *et al.*[13], more recently reestablished by Demacker *et al.*[66] that the disappearance of chylomicrons from plasma in homozygous Watanabe heritable hyperlipidemaemic rabbits which lack functional LDL receptors, is normal. In recent studies in which mice lacking the LDL receptor were used, it was shown that the initial hepatic removal of chylomicron-remnants is unaffected and therefore not mediated by the LDL receptor.[67]

Our present data are thus consistent with our earlier conclusion that normal rat hepatocytes express few if any LDL receptors.[37,68,69] It was recently suggested that proteoglycans may form the initial recognition site for lipoprotein remnants.[11] However, treatment of rat liver parenchymal cells with heparinase and/or chondroitinase did not affect ß-VLDL binding.[17,18] Furthermore, aminopeptidase M-treated lactoferrin is a very efficient inhibitor of lipoprotein remnant recognition[6] whereas it does not bind to proteoglycans.[17,18] In conclusion, the aforementioned properties point to an apoE-specific recognition site for lipoprotein remnants[70] which differs from the α_2-MR/LRP, proteoglycans or the LDL receptor. The further characterization of the nature of the tentative lipoprotein remnant receptor still offers an intriguing challenge for future research.

ACKNOWLEDGEMENT

GST-RAP fusion protein was obtained from Dr D.K. Strickland (Biochemistry Laboratory, American Red Cross, Rockville, Maryland, U.S.A), who is gratefully thanked.

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Chapter 9

General discussion and perspectives

9.1. Introduction

During thrombolytic therapy urokinase-type plasminogen activator (u-PA) is infused in order to dissolve unwanted blood clots that interrupt the blood flow and thereby exclude organs from oxygen, leading to life threatening events such as coronary thrombosis, pulmonary embolism and stroke. A drawback of the use of u-PA as a thrombolytic agent is its rapid clearance from the circulation, predominantly by the liver[1,2], leading to half-lifes ranging from 1-8 minutes depending on the species and the type of u-PA used.[1-5] As a consequence of this, in the clinic large amounts of u-PA have to be administered for a long period of time in order to obtain dissolution of the blood clot.

The studies reported in this thesis describe the mechanism of u-PA clearance by the identification of the hepatic receptors that are involved in the recognition of human u-PA. Receptors for u-PA that were known at the time point of the start of the investigation were the u-PA Receptor (uPAR)[6] that is present on e.g. monocytes, monocyte-like tumor cell lines and vascular endothelial cells.[6-8] A second receptor recognizing u-PA and u-PA:PAI-1[9-11] complexes was at that time point just identified and is named the Low Density Lipoprotein Receptor-related protein (LRP). In chapter two, differences in parenchymal liver cell recognition of two types of u-PA are described that form the base for the further investigations described in this thesis. It is shown that the liver parenchymal cell recognition of u-PA is dependent on the presence or absence of the complex carbohydrate moiety of u-PA. Therefore, the physiological fate and pharmacological application may depend on the source of u-PA used.

9.2. Liver recognition of recombinant scu-PA

DNA technology allows the production of u-PA proteins in *E.coli* bacteria by an expression vector coding for the u-PA sequence (= recombinant u-PA). In this way large amounts of u-PA can be obtained, which may reduce the costs of thrombolytic therapy. We have studied the clearance mechanism of human recombinant scu-PA (rscu-PA) in rats and determined that the liver was the major organ responsible for the recognition of rscu-PA (a maximal liver uptake of 50 % of the injected dose was reached at 5 minutes after injection of rscu-PA). We

showed that LRP was mainly responsible for the liver uptake of rscu-PA by the parenchymal liver cells and for 60 % of the Kupffer cell recognition (chapter 2, 3). For the residual Kupffer cell uptake uPAR is involved. Although the liver exists only for 2.5 % of Kupffer cells[12] (based upon protein contribution) these cells contribute for 50 % to the liver uptake of rscu-PA indicating a major role for the Kupffer cells in the recognition of rscu-PA. Parenchymal liver cells that account for 92.5 % to the liver protein contributed for 40 % to the liver uptake of rscu-PA.

Due to the described species specificity for the recognition of u-PA by uPAR (chapter 3) the contribution of human uPAR on Kupffer cells in the human liver uptake of human rscu-PA is likely to be underestimated. The occurrence of species specificity in the recognition of human u-PA by rat LRP and in reverse, has never been reported.

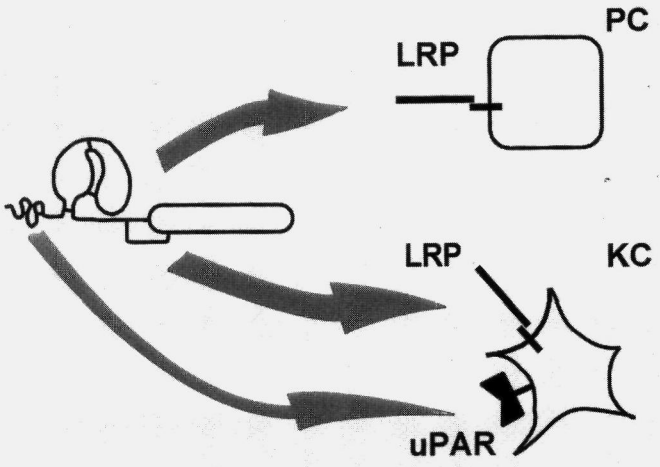

Figure 1. Schematic representation of the hepatic receptors responsible for the clearance of recombinant scu-PA produced by E.coli. Rscu-PA is recognized via residue 4-43 by the u-PA Receptor (uPAR) present on the Kupffer cells (KC) and by the Low Density Lipoprotein Receptor-related Protein (LRP) present on parenchymal (PC) and Kupffer cells. Recognition by LRP occurs via sites both within the Amino Terminal Fragment and protease domain of rscu-PA.

A model for the interaction of rscu-PA with the liver is described in figure 1. Rscu-PA is recognized via residue 4-43[13,14] by uPAR present on the Kupffer cells and by LRP present on parenchymal and Kupffer cells. Recognition of rscu-PA by LRP occurs both via sites within both the Amino Terminal Fragment (residue 1-

135) and protease domain (residue 136-411) of u-PA as has been proposed by Nykjaer *et al.*[15] Deletion of residue 11-135 (=delta 125-rscu-PA) abolishes the interaction with uPAR and a part of the recognition site(s) necessary for high affinity recognition by LRP, leaving only a low affinity binding to LRP thereby reducing the liver uptake. In chapter two it is described that the binding of rscu-PA to LRP on isolated parenchymal liver cells can not be competed for by delta125-rscu-PA. It was also observed that ^{125}I-delta125-rscu-PA did show hardly any binding to isolated parenchymal liver cells. From this it can be concluded that at least two sites within u-PA are necessary for high affinity binding to LRP and upon removal of one of the sites low affinity binding is left. *In vivo* residual binding of delta125-rscu-PA to LRP was observed by the complete reduction of the liver association by preinjection of RAP.

9.3. Liver recognition of glycosylated tcu-PA

9.3.1. Recognition of tcu-PA by the asialoglycoprotein receptor and heparan sulfate proteoglycans

Natural sources of human u-PA are plasma and urine. Since plasma contains 6-38 times less u-PA than urine, commercially available u-PA preparations are often extracted from human urine. About 72 % of the u-PA present in urine is in an active HMW-tcu-PA form.[16-19] In chapter 4-6 the receptors responsible for the liver uptake of urinary tcu-PA are identified and a model for the hepatic uptake of urinary tcu-PA is depicted in figure 2. The identification of the asialoglycoprotein receptor (ASGPr) as a receptor contributing to the recognition of glycosylated urinary human tcu-PA was determined by isolation of the receptor from human and rat liver using a tcu-PA-Sepharose column. In addition binding of tcu-PA to the ASGPr was shown in a purified system, as well as by *in vivo* experiments in rats and *in vitro* experiments using freshly isolated rat parenchymal liver cells (chapter 4, 5). An additional control was performed by culturing freshly isolated rat parenchymal liver cells for 2 days. It is known that upon culturing, the expression of the ASGPr is completely downregulated. This results in the loss of association of urinary tcu-PA (and the control ligand ASOR) to these cells (data not shown).

It is established that urinary tcu-PA is mainly recognized by the parenchymal liver cells by the (parenchymal liver cell specific) asialoglycoprotein receptor and by heparan sulfate proteoglycans (HSPGs, (chapter 4)). The HSPGs interaction of tcu-PA was established by the *in vivo* and *in vitro* inhibition of the liver interaction of tcu-PA by the HSPGs antagonist protamine sulfate. The complex carbohydrate of the tcu-PA preparation is a mixture of sulfated and non-sulfated GalNAcß1-4GlcNAcß1-2Man terminal bi- and triantennary glycans.[20] As a result of this, the tcu-PA mixture is recognized by different systems by the parenchymal liver cells

(chapter 5, 6). Non-sulfated, GalNAc-terminal glycosylated tcu-PA (30 % of the preparation) is mainly recognized by the ASGPr (figure 2 A). Non-sulfated tcu-PA is recognized by HSPGs probably via a heparin binding site that has been identified in the kringle and protease domain.[21,24] Due to sulfation of the terminal GalNAcs at the complex carbohydrate of urinary tcu-PA (70 % of the tcu-PA preparation), recognition of sulfated tcu-PA by the ASGPr was prevented and only recognition by HSPGs occurred (figure 2B). A minor amount of sulfated and non-sulfated tcu-PA is recognized by yet to be identified other proteoglycans or receptor(s) on the liver.

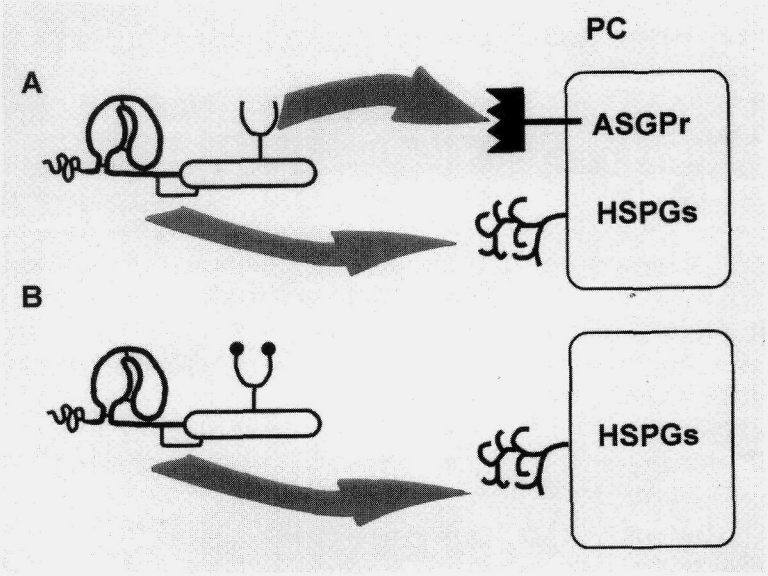

Figure 2. Schematic representation of the hepatic receptors responsible for the clearance of glycosylated tcu-PA obtained from human urine. Non-sulfated tcu-PA (A) is predominantly recognized via its carbohydrate side chain by the asialoglycoprotein receptor (ASGPr) and less prominent via its protein moiety by heparan sulfate proteoglycans (HSPG) on parenchymal liver cells (PC). Sulfated tcu-PA (B) is prevented from recognition by the asialoglycoprotein receptor and is only recognized by heparan sulfate proteoglycans. ⊃— ,GalNAc-terminal biantennary complex carbohydrate; ●, SO_4.

Tcu-PA is recognized by heparan sulfate proteoglycans (HSPGs) on the parenchymal liver cells, which was suggested before by Strickland *et al.*[23] on the base of the strong heparin binding properties of the kringle domain[24] of u-PA.

Rijken *et al.*[21] showed that heparin binding of u-PA also occurs via the protease domain of u-PA. Due to the collagenase isolation procedure the parenchymal liver cells used during the experiments are devoid of a large part of their extracellular proteoglycans. This is the reason for a discrepancy between our *in vitro* and *in vivo* data. The interaction of tcu-PA with HSPGs on parenchymal liver cells is barely detectable *in vitro* while *in vivo* HSPGs interactions are observed (chapter 4). Sulfated tcu-PA (isolated by WFA-Sepharose, chapter 6) that is only recognized by proteoglycans shows low binding to isolated parenchymal liver cells, whereas *in vivo* the interaction with proteoglycans in the liver is obvious. The use of isolated parenchymal cells in culture (with a restored extra cellular matrix) is not preferred since it has been described that culturing of parenchymal cells induces the expression of proteoglycans other than present in the liver *in vivo*.[25]

Upon isolation of the ASGPr from human and rat liver by a tcu-PA-Sepharose column, proteins were detected ranging from 40-60, 90-110, >150 kDa (chapter 4, figure 1, 2). By means of immunoblotting with anti-rat ASGPr antibodies it was shown that these proteins represent the rat ASGPr subunits RHL-1-3 (44, 49 and 60 kDa), some dimers (90-110 kDa) and other multimers (>150 kDa). Most, but not all of the multimers, disappeared under reducing conditions indicating that some of the RHL-multimers were stable upon treatment with the reducing agent ß-mercaptoethanol. It has never been described that disulfide bridges are formed between RHL subunits of the ASGPr[22], therefore probably non-covalent forces keep the multimers together.

The observation by Henkin *et al.*[26] described in chapter 1.4 that the sialic content of glycosylated HMW-scu-PA determines its plasma clearance in rabbits can be explained by the knowledge obtained in this thesis. More sialic acid protects the terminal Gal or GalNAc of the complex carbohydrate, and thereby the recognition by the ASGPr is abolished and concurrently the clearance is delayed. The differences in clearance between the u-PA preparations described by Henkin *et al.*[26] are also confirmed by our data that show the recognition of Sp2/0 derived- (Abbott), human kidney cell culture derived- (Abbott) and transformed human kidney cell culture produced HMW-scu-PA (Sandoz) by the ASGPr on rat parenchymal liver cells and purified ASGPr. CHO derived LMW-scu-PA (Leuven) was not recognized by the ASGPr (chapter 5 (purified ASGPr) and data not presented in this thesis (*in vitro* data)).

From the results obtained with human kidney cell derived glycosylated HMW-scu-PA (chapter 2) it was concluded that the 'lectin like receptor' responsible for the parenchymal liver cell uptake of HMW-scu-PA was likely to be the asialoglycoprotein receptor since the binding characteristics of glycosylated u-PA resembled the characteristics for binding to the ASGPr[22]: calcium-dependent, high affinity binding to parenchymal liver cells that could be inhibited by lactose and glycosylated LMW-tcu-PA. Furthermore, removal of the carbohydrate side chain by treatment of u-PA with the enzyme N-glycosidase F abolished its competitive

ability. The ASGPr antagonist ASOR indeed showed complete inhibition of the binding thereby confirming the interaction with the ASGPr (not shown). This is in contrast to results published before by Kuiper *et al.*[27] who used the same kidney cell derived HMW-scu-PA but could not identify the ASGPr as the hepatic receptor. It was concluded from their results that HMW-scu-PA was recognized via the protease domain (that contains the complex carbohydrate side chain), since LMW-scu-PA (containing the connecting peptide and protease domain of u-PA) showed the same binding characteristics as HMW-scu-PA and was able to compete for the binding of HMW-scu-PA. An explanation may be that the batch of HMW-scu-PA they used differed from the batch used in the experiments described in chapter 2 in for instance the amount of sulfation of the complex carbohydrate chain of HMW-scu-PA.

LRP that mediates the hepatic uptake of *E.coli* produced human rscu-PA did not play a role in the uptake of urinary human tcu-PA even when the complex carbohydrate was removed by enzymatic treatment with N-glycosidase F (chapter 2, 4, 6). It is thought that the different sources of u-PA (human urine or *E.coli*) may be responsible for this. Probably differences in folding of the protein exist between *E.coli* produced and urinary tcu-PA. Twelve disulfide bridges have to be formed, one disulfide bridge that is out of place (thereby disturbing 4 cysteines) can already lead to a different folding of the protein. Denaturing and subsequent renaturing of the recombinant protein during isolation[28] is performed to overcome misfolding of the protein but still this is not a guarantee that the overall structure exactly resembles the u-PA protein obtained from urine or plasma. Furthermore, the u-PA protein may also be phosphorylated at its protein core.[29-31] Differences in the extent or pattern of phosphorylation can not be excluded to be a source of differences in receptor recognition. Finally an explanation for the observed difference in recognition by LRP could be the difference in single-chain or two-chain form of the preparations. It has been described by Kasai *et al.*[32] that due to activation of u-PA the conformation of the protein is changed. One can imagine that scu-PA is recognized by LRP but that due to activation and change in conformation LRP recognition sites within tcu-PA have disappeared.

Since the urinary tcu-PA preparation is a mixture of proteins with differently sulfated complex carbohydrate side chains, that determine receptor recognition, the question remains whether one individual possesses the same mixture of sulfated carbohydrates or that the mixture is due to the large pool of urine used obtained from different individuals having different patterns of sulfation or that in urine all the carbohydrates are sulfated but that, due to isolation procedures, sulfate is lost.

Other organs besides the liver also contributed to the rapid plasma clearance of u-PA. The kidneys, muscles and skin were responsible for about 16 % of the extra hepatic clearance. The interaction of u-PA with these organs is probably mediated via proteoglycans present on the endothelial cells of these organs since no inhibitory effect of GST-RAP was observed thereby excluding the involvement

of LDL receptor family members in the extrahepatic uptake (chapter 3). The ASGPr can not be responsible since this receptor only occurs on the parenchymal liver cells. An indication for a role of proteoglycans in the extrahepatic uptake was obtained by the observation described in chapter 4 that upon injection of the heparan sulfate proteoglycan antagonist protamine sulfate the interaction of tcu-PA with the kidneys and skin was dramatically reduced. Proteoglycans on the vascular endothelial cells may also play a role in the attachment of u-PA.

9.3.2. Implications in hepatic recognition when tcu-PA is complexed with PAI-1

Chapter 7 shows that complex formation of tcu-PA with its physiological inhibitor PAI-1 results in the initiation of recognition of the tcu-PA:PAI-1 complex by LRP via sites within PAI-1 (Lysine 69)[33] and probably tcu-PA. It was shown that the recognition sites were "cryptic sites" that are exposed upon complex formation. This is in agreement with the observation in chapters 4-6 that uncomplexed tcu-PA is not recognized by LRP, and with the idea that recombinant scu-PA is recognized by LRP in a different conformation compared to tcu-PA. Horn *et al.*[33] recently described the presence of a cryptic LRP binding site in the heparin binding domain of PAI-1 (Lysine 69) that is not present in PAI-1 but is exposed upon complex formation of PAI-1 with tissue-type plasminogen activator (t-PA). They showed that this Lysine 69 was responsible for the part of the recognition of t-PA:PAI-1 by LRP via the PAI-1 moiety. Our results indicate that the same Lysine 69 is also involved in the interaction of tcu-PA:PAI-1 with LRP. *In vivo* studies confirmed the importance of Lysine 69 in the LRP recognition of t-PA:PAI-1 complexes.

The general idea that LRP is thought to be a receptor for u-PA:PAI-1 rather than for the free components (as stated in chapter 7) is confirmed by the results obtained with uncomplexed and complexed tcu-PA with PAI-1. The recognition of *E.coli* produced recombinant scu-PA, mainly by LRP, seems to be an exception to the rule but can be explained by a different conformation of the rscu-PA and urinary tcu-PA. Rscu-PA already exposes the cryptic sites necessary for recognition by LRP and lacks the complex carbohydrate side chain and therefore recognition by the ASGPr. It would be interesting to study whether complex formation of rtcu-PA with PAI-1 further increases the affinity for LRP.

9.4. On the improvement of thrombolytic therapy with u-PA

An approach to improve thrombolytic therapy with u-PA is the inhibition of the rapid clearance by the specific blockage of the uptake of u-PA by the liver. Our studies showed that several receptors within the liver are responsible for the rapid clearance of u-PA. With this knowledge it may be possible to develop a strategy to prolong the half-life of u-PA during thrombolytic therapy.

The interaction with the ASGPr can be specifically inhibited by the high affinity antagonist cluster galactoside TrisGal(20Å).[34] The ASGPr is only present on

the parenchymal liver cells, therefore, blockade of the interaction with this receptor abolishes all the interactions mediated via the GalNAc terminal carbohydrate of u-PA. The clearance of other proteins that are recognized by the ASGPr can however also be influenced although another physiological substrate for the receptor is uptill now not yet identified. The inhibition of the interaction of u-PA with LRP was brought about during our studies with GST-RAP. GST-RAP is not a suitable candidate to use in the clinic since (1) it inhibits the binding of multiple ligands to LRP (table 1, chapter 1) that are endogenously present in the circulation and (2) GST-RAP also binds to other LDL Receptor family members. This may lead to unwanted side effects by the disarrangement of several important processes in the body. The side effects can be prevented by the development of a recombinant protein that mimics the 6th, 7th and 8th complement repeat of the second cluster of complement-type repeats of LRP since it was shown by Horn *et al.*[35] that this sequence possesses the u-PA binding site. The possibility that other ligands also are recognized by this part of the LRP protein can be overcome by a further reduction of the u-PA binding sequence in LRP to finally end up with a small u-PA-specific binding sequence. A second option is to search in the u-PA molecule for the sequences that are recognized by LRP and use these sequences as antagonist, or third, to search in the RAP protein for the sequence that inhibits only the binding of u-PA to LRP and use this RAP-sequence as specific inhibitor. Finally antibodies can be generated that specifically inhibit the interaction of u-PA to LRP. It is not known which sequences within the u-PA molecule are responsible for the binding to LRP. Our data indicate a major role for the ATF domain of u-PA since the deletion mutant delta125-rscu-PA was poorly recognized by the liver. But as described by Nykjaer *et al.*[15] and Andreassen *et al.*[36] sites within the protease domain of u-PA are of importance too for the binding to LRP. For the inhibition of the interaction of u-PA with proteoglycans probably the kringle domain of u-PA is of major importance.[24] This can be prevented by using a mutant u-PA that lacks the kringle domain or by the use of the isolated kringle domain as inhibitor. One should keep in mind that the specific receptor antagonists should not be of such a small size that rapid renal filtration occurs. To overcome this, the protein can be coupled to a stabilizing, neutral, non-receptor recognized large protein such as BSA.

From the obtained information on the hepatic recognition of u-PA provided in this thesis it can be deduced that for the use in thrombolytic therapy LMW-scu-PA is a promising candidate. This has also been suggested by Spriggs, Stump and de Munk.[37-39] By the removal of the epidermal growth factor domain of HMW-scu-PA, the interaction with uPAR is prevented. Deletion of the kringle domain probably prevents the interaction with proteoglycans via its major heparin binding site. Furthermore, the interaction of HMW-scu-PA with LRP is disturbed. This leaves the source of which the u-PA is obtained to a determining factor in the liver recognition. It has been described by Lenich *et al.*[40] that the observation that *E.coli*

produced rscu-PA is more active in fibrinolysis than glycosylated scu-PA[41,42] is caused by the presence or absence of the complex carbohydrate at Asn_{302} and probably by conformational differences. For this reason *E.coli* produced LMW-scu-PA (such as the delta125-rscu-PA used in chapter 2, 3) is a more suitable candidate for thrombolytic therapy. It was shown that the liver uptake of delta125-rscu-PA can be completely abolished by the inhibition of the interaction with LRP by injection of GST-RAP. Therefore thrombolytic therapy may be improved by the preinjection of an as aforementioned specific inhibitor of the interaction of u-PA with LRP. When glycosylated LMW-scu-PA is used, the interaction with the asialoglycoprotein receptor and proteoglycans has to be interrupted to abolish liver uptake.

9.5. Perspectives

By the identification of the Low Density Lipoprotein Receptor-related Protein (LRP), the asialoglycoprotein receptor (ASGPr) and heparan sulfate proteoglycans (HSPGs) as hepatic receptors responsible for the rapid clearance of u-PA, the inhibition of the rapid elimination of u-PA from the circulation by the liver may be realized by the, as in chapter 9.4 described, optimalization or development of specific inhibitors for the interaction of u-PA with LRP, ASGPr and HSPGs. A combination of different approaches might be needed to improve the therapeutic use of u-PA as a thrombolytic agent.

The aforementioned approach could also be used to increase the plasma level of endogenous u-PA, which can be beneficial for patients with an increased risk of thrombosis. Because the exact composition and structure of the complex carbohydrate of human endogenous u-PA is not known, it is interesting to investigate this and the consequences of the interactions with the liver receptors. Determination of the effect of the increase of endogenous u-PA in the circulation on thrombus formation may provide information on the use of endogenous u-PA as a strategy for antithrombotic therapy.

Proteoglycans may contribute to the binding of endogenous u-PA associated to vascular endothelial cells and might thereby form an important intravascular pool of u-PA. Inhibition of the association of endogenous u-PA to the vascular wall may be beneficial for haemostasis. It will be of interest to further elucidate the interaction of u-PA with the proteoglycans. This can be obtained by isolation of proteoglycans from liver and vascular endothelial cells and quantification of their affinity for u-PA. The proteoglycan(s) that specifically bind to u-PA can be purified by means of affinity chromatography and further characterized for e.g. amino acid composition, molecular weight. Knowledge on the interaction of u-PA with proteoglycans may lead to new strategies to improve thrombolytic therapy.

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Chapter 10

Summary

Thrombolytic therapy with the use of urokinase-type plasminogen activator (u-PA) is hampered by the rapid disappearance of the thrombolyticum from the circulation due to a rapid uptake by especially the liver. In this thesis the receptors responsible for the liver uptake of u-PA were identified. With the obtained knowledge it may be possible to develop specific inhibitors for the interaction of u-PA with the liver, leading to an increased amount of u-PA in the circulation. This will lead to an improved thrombolytic therapy.

Chapter 2 describes the recognition sites for two types of human high molecular weight single-chain urokinase (HMW-scu-PA) by rat parenchymal liver cells. Native, glycosylated HMW-scu-PA produced by a human kidney cell culture is recognized via its carbohydrate moiety by a 'lectin like' recognition site, since removal of the complex carbohydrate present at Asn_{302} reduced the parenchymal liver cell binding to the level of non-specific binding. Furthermore, deglycosylated high molecular weight two-chain u-PA (HMW-tcu-PA) was able to compete only for 25 % of the binding of glycosylated ^{125}I-HMW-scu-PA while glycosylated HMW-tcu-PA was able to compete for 75 % of the binding of ^{125}I-HMW-scu-PA. The binding of HMW-scu-PA to the parenchymal liver cells was dependent on calcium and could be inhibited by lactose. *E.coli* produced recombinant scu-PA (rscu-PA) is not recognized by the 'lectin like' receptor since it lacks, due to its production in *E.coli,* the complex carbohydrate moiety. Recombinant scu-PA was shown to be recognized by the Low Density Lipoprotein Receptor-related Protein (LRP) since glutathione-S-transferase coupled Receptor-Associated Protein (GST-RAP), the universal antagonist of LRP, effectively inhibited the binding of rscu-PA to the parenchymal liver cells.

The contribution of LRP in the liver uptake and plasma clearance of rscu-PA *in vivo* was further investigated in chapter 3. Upon injection into rats, rscu-PA is rapidly cleared from the circulation and taken up by the liver. A maximal liver uptake of 50 % was reached at 5 min after injection. The parenchymal and Kupffer cells contributed for 40 and 50 % to the liver uptake, respectively. The involvement of LRP in the liver uptake of rscu-PA was shown by a 40 % inhibition of the liver uptake by a preinjection of 50 mg/kg body weight GST-RAP and a 1.5-fold

delayed serum clearance. The dose of GST-RAP necessary to completely inhibit the interaction with LRP was determined by using α_2-macroglobulin, since this ligand is exclusively recognized by LRP (chapter 3, 8). By the use of a deletion mutant of rscu-PA that lacks residue 10-135 it was shown that recognition by LRP occurs both via sites within residue 136-411 and 1-135. Furthermore, the involvement of the u-PA Receptor (uPAR) in the Kupffer cell recognition of rscu-PA was shown by a reduction in the Kupffer cell interaction of rscu-PA upon pretreatment of the Kupffer cells with the enzyme PI-PLC that removes GPI-linked proteins like uPAR. It was concluded that *in vivo* LRP is responsible for more than 90 % of the parenchymal liver cell mediated uptake of rscu-PA and for 60 % of the Kupffer cell interaction. For the residual Kupffer cell recognition uPAR is likely to be involved.

In chapter 8 data are shown that demonstrate the binding characteristics of LRP *in vitro* and *in vivo* by using α_2-macroglobulin (activated with trypsin) and GST-RAP. GST-RAP is rapidly cleared from the circulation and taken up by the liver, which is coupled to efficient intracellular degradation. The association and degradation of α_2-macroglobulin by parenchymal liver cells at 37 °C was inhibited by RAP and lasted for 1-2 hour.

The receptor responsible for the liver recognition of glycosylated HMW-tcu-PA obtained from human urine was isolated from human and rat liver by means of a tcu-PA-Sepharose column (chapter 4). By using antibodies against the asialoglycoprotein receptor (ASGPr) it was determined that this receptor is the receptor responsible for the recognition of glycosylated tcu-PA. *In vitro* the recognition of tcu-PA by the ASGPr on parenchymal liver cells was shown by the inhibition of the binding of tcu-PA by ASOR, an ASGPr antagonist, and the antibodies against the ASGPr. Upon injection into rats, glycosylated tcu-PA was rapidly cleared from the circulation and taken up by the liver for maximally 40 % of the injected dose. The involvement of the ASGPr in the liver uptake *in vivo* was shown by a preinjection of an excess of ASOR or enzymatic removal of the complex carbohydrate of tcu-PA. This resulted in a 42 % reduction of the maximal liver uptake. Besides by the ASGPr, it was determined that tcu-PA was also recognized by heparan sulfate proteoglycans (HSPGs) on the parenchymal liver cells.

The carbohydrate moiety of urinary tcu-PA is a mixture of sulfated and non-sulfated GalNAcß1-4GlcNAcß1-2Man terminal bi- and triantennary glycans. To investigate whether sulfation determines the interaction with the cellular binding sites ASGPr and HSPGs, sulfated and non-sulfated tcu-PA were separated by means of a Wisteria floribunda agglutinin Sepharose column (chapter 5). Data in chapter 5 and 6 obtained with an *in vitro* binding assay using purified ASGPr, and by binding of tcu-PA to the ASGPr on parenchymal liver cells *in vitro* and *in vivo* showed that sulfated tcu-PA was recognized by HSPGs while non-sulfated tcu-PA

was recognized by the ASGPr. Removal of the carbohydrate moiety of non-sulfated tcu-PA or inhibition of the interaction with the ASGPr by addition of the antagonist ASOR, abolished the recognition by the ASGPr and resulted in recognition of tcu-PA by HSPGs.

Chapter 7 shows that complex formation of tcu-PA with its physiological inhibitor plasminogen activator inhibitor type-1 (PAI-1) resulted in a 2-fold enhanced liver uptake and a 1.6-fold increased serum clearance. The enhanced liver uptake of tcu-PA:PAI-1 compared to tcu-PA was shown to be caused by recognition of the tcu-PA:PAI-1 complex by LRP since the LRP antagonist RAP reduced the liver uptake 2.2-fold. The ASGPr and HSPGs still were responsible for a part of the liver uptake of tcu-PA:PAI-1. The recognition of tcu-PA:PAI-1 by LRP was further studied by using mutants of PAI-1 that possessed lysine to alanine mutations in the heparin binding domain of PAI-1. The experiments showed that due to the complex formation, cryptic LRP binding sites both within tcu-PA and PAI-1 (lysine 69) were exposed on the protein surface that are not present in uncomplexed tcu-PA and PAI-1 and that these cryptic sites were responsible for the recognition of tcu-PA:PAI-1 by LRP.

In conclusion, the liver recognition of u-PA is dependent on the source of which u-PA is isolated from. Depending on the protein folding, the presence of a complex carbohydrate moiety at Asn_{302} with terminal GalNAc residues that may have bound sulfate, and complex formation with PAI-1, liver recognition occurs via the Low Density Lipoprotein Receptor-related Protein, u-PA Receptor, asialoglycoprotein receptor or heparan sulfate proteoglycans.

Chapter 11

Samenvatting voor niet-ingewijden

De vloeibaarheid van het bloed is het resultaat van de balans tussen stolling en fibrinolyse (oplossen van stolsels). Wanneer het evenwicht tussen stolling en fibrinolyse verstoord is, kan dit leiden tot ongewenste stolsels in het bloed (trombose). Hierdoor kunnen organen niet meer worden voorzien van voldoende zuurstof wat mogelijk kan leiden tot een levensbedreigende situatie. Bloedstolsels kunnen een bloedvat afsluiten en afhankelijk van het weefsel leidt dit tot een hartaanval, een beroerte of andere vormen van trombose. Bloedstolsels kunnen opgelost worden door een enzym genaamd plasmine dat in het stolsel aanwezig fibrine kapot knipt (fibrinolyse). Dit plasmine moet echter eerst actief gemaakt worden door zogenaamde plasminogeen activatoren. Eén van de in het menselijk lichaam voorkomende plasminogeen activatoren is urokinase-type plasminogeen activator (u-PA). U-PA wordt in de kliniek therapeutisch toegepast om stolsels op te lossen (= thrombolytische therapie).

Een probleem bij van het gebruik van u-PA voor thrombolytische therapie is dat u-PA erg snel uit het bloed verdwijnt waarvoor de lever voornamelijk verantwoordelijk is. Hierdoor moeten patiënten langdurig een grote hoeveelheid u-PA toegediend krijgen teneinde de stolsels volledig op te lossen. Dit brengt hoge kosten met zich mee. Het promotieonderzoek richtte zich op de identificatie van de receptoren op de lever die verantwoordelijk zijn voor de snelle verwijdering van u-PA uit het bloed. Receptoren zijn eiwitten op het oppervlak van cellen die andere stoffen (liganden) zoals suikers en eiwitten (zoals u-PA) herkennen en binden. De levercellen kunnen deze receptoren vanaf hun oppervlak opnemen waarna in de levercel de receptor het u-PA loslaat en zelf weer terug gaat naar het celoppervlak om opnieuw u-PA uit de bloedbaan te binden. In de cel wordt het u-PA afgebroken. Met de verkregen kennis van de leverherkenning van u-PA kan de thrombolytische therapie verbeterd worden door de binding van u-PA aan de leverreceptoren te verhinderen. Hierdoor kan het toegediende u-PA langer in de bloedbaan blijven circuleren, meer plasminogeen omzetten tot het actieve plasmine waardoor de bloedstolsels beter opgelost worden.

Eiwitten (zoals u-PA) bestaan uit een ketting van bouwstenen, aminozuren genaamd. Het u-PA eiwit kan in een aantal verschijningsvormen voorkomen. u-PA wordt gemaakt als een enkele keten aminozuren (= single-chain u-PA = scu-PA).

Plasminogeen wordt door dit scu-PA langzaam geactiveerd tot plasmine. Pas als er een knip in het scu-PA wordt aangebracht is het in staat om plasminogeen snel te activeren. Dit actieve u-PA wordt two-chain (twee-'ketenig') u-PA genoemd (tcu-PA). Het u-PA eiwit is opgebouwd uit 411 aminozuren. De eerst 135 aminozuren worden ook wel ATF-u-PA genoemd (ATF = Amino Terminaal Fragment). Aminozuur 136-411 vormen het LMW-u-PA (LMW = Laag Moleculair geWicht). Urokinase kan opgezuiverd worden uit bloed of urine. Voorts kunnen gekweekte niercellen u-PA produceren. Deze u-PA's hebben een ingewikkelde suikerstructuur aan het eiwit gebonden waarvan de uiteinden bestaan uit galactose of galactose-derivaten waaraan vervolgens sulfaat of siaalzuur gebonden kan zijn. Nieuwe ontwikkelingen op het gebied van DNA-technologie maken het mogelijk om het gen van scu-PA in een bacterie te brengen en deze bacterie grote hoeveelheden van het scu-PA eiwit te laten maken (= recombinant scu-PA, rscu-PA). Een gevolg van deze methode is dat er geen suikers op het eiwit gezet kunnen worden en dat de opvouwing van het eiwit anders kan zijn.

Voor de bestudering van de herkenning van u-PA door receptoren op de lever zijn rattelevers gebruikt waaruit de levercellen geisoleerd zijn. De lever is opgebouwd uit een aantal verschillende celtypen: de parenchym-, endotheel- en Kupffercellen. De parenchymcellen komen het meest voor, 92,5 % van de lever bestaat uit deze cellen. Ook werd radioactief u-PA in de bloedbaan van ratten ingespoten en werd de totale leveropname bestudeerd.

In hoofdstuk 2 wordt het verschil in parenchymcel herkenning beschreven van twee soorten u-PA. Aangetoond werd dat de leverherkenning afhankelijk is van de aanwezigheid van de suikergroep op het u-PA eiwit. Suikerloos rscu-PA (gemaakt door *E.coli* bacteriën) bindt aan lever parenchym- en Kupffer cellen (hoofdstuk 2, 3). 60 % van het u-PA wat aan de Kupffercellen bindt en meer dan 90 % van het rscu-PA wat aan de parenchymcellen bindt wordt herkend door de receptor genaamd 'Low Density Lipoprotein Receptor-related Protein' (LRP). De herkenning door LRP werd bestudeerd door gebruik te maken van het eiwit 'Receptor-Associated Protein' (RAP). RAP bindt aan LRP en voorkomt dat andere liganden, zoals rscu-PA, aan LRP binden. De verwijdering van rscu-PA uit het bloed (= klaring) door opname via LRP in lever werd bestudeerd door de injectie van radioactief rscu-PA in de bloedbaan. Een snelle klaring uit het bloed (in minder dan 1 min is al 50 % uit het bloed verwijderd) en een snelle opname van het rscu-PA door de lever werd bepaald (hoofdstuk 3). Herkenning van rscu-PA door LRP werd aangetoond door 1 minuut voordat rscu-PA wordt ingespoten een grote hoeveelheid RAP in te spuiten. RAP bindt eerst aan LRP en voorkomt hiermee dat rscu-PA aan LRP kan binden. Door het remmen van de binding van rscu-PA aan LRP wordt de leveropname van rscu-PA met 40 % verlaagd en blijft het rscu-PA langer in de bloedbaan. Om een indruk te krijgen welk deel van rscu-PA herkend wordt door LRP werd een mutant rscu-PA ingespoten wat de

aminozuren 10-135 mist (delta125-rscu-PA). Delta125-rscu-PA bleek alleen door LRP herkend te worden want RAP blokkeert de lever opname volledig. In hoofdstuk 8 is de interactie van RAP met LRP verder bestudeerd. Hierbij werd gebruik gemaakt van α_2-macroglobuline. Dit eiwit bindt alleen maar aan LRP en is als model gebruikt ter bestudering van de interactie met LRP en de remmende werking van RAP en RAP was dan ook instaat om de lever opname van α_2-macroglobuline door LRP volledig te remmen. RAP blokkeert niet de hele leveropname van rscu-PA. Daarom werd verondersteld dat nog een receptor een rol speelt in de lever herkenning. Aangetoond werd dat de binding van rscu-PA aan de Kupffer cellen 40 % gevoelig is voor behandeling van de cellen met het enzym phosphatidylinositol phospholipase C (PI-PLC). Hieruit werd geconcludeerd dat rscu-PA op de Kupffer cellen naast LRP ook herkend wordt door de u-PA Receptor (uPAR).

De in hoofdstuk 2 en 3 beschreven studies tonen aan dat LRP verantwoordelijk is voor meer dan 90 % van de parenchymcel herkenning en voor 60 % van de Kupffercel herkenning van *E.coli* geproduceerd rscu-PA. Voorts zijn er aanwijzingen voor de binding van rscu-PA aan de u-PA Receptor (uPAR) op de Kupffer cellen.

In hoofdstuk 2 wordt beschreven dat geglycosyleerd scu-PA afkomstig van een niercel cultuur niet door LRP herkend wordt maar dat herkenning optreedt via de suiker groep. Verwijdering van de complexe-type suikerketen van het scu-PA oppervlak door behandeling met het enzym N-glycosidase F resulteerde in het verlies van de binding aan parenchymcellen. Omdat de binding van scu-PA aan parenchymcellen voorts calcium vereist en te remmen was door de suiker lactose werd geconcludeerd dat uit niercellen afkomstig geglycosyleerd scu-PA herkend wordt door een suiker-bindende receptor.

Geglycosyleerd two-chain u-PA (tcu-PA) afkomstig uit urine blijkt op een vergelijkbare manier door de lever parenchymcellen herkend te worden als niercel scu-PA (hoofdstuk 4). De receptor verantwoordelijk voor de herkenning van geglycosyleerd tcu-PA werd opgezuiverd uit levers van rat en mens. Door gebruik te maken van antilichamen die de asialoglycoproteine receptor (ASGPr) herkennen (een suiker receptor die in het lichaam alleen voorkomt op lever parenchymcellen), werd aangetoond dat deze receptor verantwoordelijk is voor de herkenning van geglycosyleerd tcu-PA (hoofdstuk 3). De ASGPr herkent galactose suikergroepen of galactose-derivaten. Urinair tcu-PA heeft op het uiteinde van zijn suikerketen twee of drie vrije galactose-derivaten waardoor herkenning via de ASGPr kan plaatsvinden. Op parenchymcellen is de herkenning van geglycosyleerd tcu-PA door de ASGPr aangetoond door het verdwijnen van de tcu-PA binding door het toevoegen van ASOR, een competatieve verbinding met veel galactoses aan het oppervlak. De bijdrage van de ASGPr in de klaring van geglycosyleerd urinair tcu-PA is bestudeerd door preinjectie van een grote hoeveelheid ASOR. Hoewel de

leveropname van tcu-PA door de preinjectie van ASOR met 42 % wordt verlaagd is er slechts een klein effect op de klaring. Er blijkt naast de ASGPr, tenminste nog één ander type receptor verantwoordelijk voor de leverherkenning van tcu-PA, namelijk heparansulfaat proteoglycanen (HSPGs). Naast herkenning door de lever wordt tcu-PA ook opgenomen door de nieren, huid en spieren.

Geconcludeerd wordt dat de asialoglycoproteine receptor en heparansulfaat proteoglycanen op de lever parenchymcellen verantwoordelijk zijn voor de snelle klaring van geglycosyleerd tcu-PA uit de circulatie.

De galactose-derivaten van de complex-type suikergroep op tcu-PA kunnen al dan niet gecamoufleerd zijn door sulfaatgroepen. Het urinaire tcu-PA bestaat uit een mengsel van wel en niet gesulfateerde galactose-derivaten. Omdat bekend is dat de ASGPr alleen vrije galactoses (-derivaten) herkent is bestudeerd of de aanwezigheid van sulfaat op de galactose-derivaten de receptorherkenning van tcu-PA beinvloedt. Hiertoe werd het urinaire u-PA preparaat gescheiden in een fractie met gesulfateerde galactose-derivaten en een fractie met vrije galactose-derivaten op het u-PA (hoofdstuk 5). Gebleken is dat niet-gesulfateerd tcu-PA vooral door de ASGPr herkend werd (hoofdstuk 5, 6). Het gesulfateerde tcu-PA werd niet door de ASGPr herkend maar alleen door HSPGs (hoofdstuk 5, 6). Hieruit werd geconcludeerd dat sulfatering van de eindstandige galactose-derivaten van de suiker van tcu-PA de receptor herkenning op de lever inderdaad bepaalt. Verhindering van de interactie met de ASGPr of sulfatering van de galactose-derivaten voorkomt de herkenning van tcu-PA door de ASGPr. Wanneer er geen herkenning door de ASGPr kan plaatsvinden wordt tcu-PA herkend door HSPGs. Het voorkomen van de interactie met de ASGPr en/of HSPGs door het inspuiten van de remmers ASOR (voor ASGPr) en protamine sulfaat (voor HSPGs) reduceert de leveropname en vertraagt de plasmaklaring waardoor er meer u-PA in het bloed aanwezig blijft.

Tenslotte is het effect van complexering van tcu-PA met zijn remmer PAI-1 bestudeerd (hoofdstuk 8). In de circulatie kan de enzymactiviteit van tcu-PA geinactiveerd worden door de binding van een eiwit genaamd Plasminogeen Activator Inhibitor type-1 (PAI-1). Hierdoor kan tcu-PA plasminogeen niet meer omzetten tot plasmine wat kan leiden tot een verminderde fibrinolyse. Omdat geglycosyleerd tcu-PA niet door LRP herkend wordt was het interessant het gevolg van complexering met PAI-1 op de leverherkenning te bepalen. Complexen van tcu-PA met PAI-1 en een aantal mutanten van PAI-1 zijn gemaakt en opgezuiverd. Studies in ratten tonen aan dat 2 maal zoveel tcu-PA:PAI-1 door de lever werd opgenomen vergeleken met ongecomplexeerd u-PA. Door de hogere lever opname werd het tcu-PA:PAI-1 complex sneller uit het serum geklaard dan tcu-PA. De verhoogde leveropname van tcu-PA:PAI-1 t.o.v. tcu-PA werd veroorzaakt door herkenning van het complex door LRP. De ASGPr en HSPGs

blijven een rol spelen in de leveropname. Door gebruik te maken van PAI-1 mutanten is aangetoond dat u-PA:PAI-1 bindt aan LRP via plaatsen in PAI-1 en tcu-PA die verborgen zijn in de losse eiwitten en aan het oppervlak komen wanneer tcu-PA en PAI-1 gecomplexeerd zijn.

In conclusie, de leverherkenning van u-PA is afhankelijk van de bron waaruit het geisoleerd wordt. Afhankelijk van de vouwing van het u-PA eiwit, de aanwezigheid van het complex-type suiker met galactose-derivaten op het uiteinde welke al dan niet sulfaat gebonden kunnen hebben en complexering met PAI-1, vindt lever opname plaats via LRP, uPAR, de asialoglycoproteine receptor en heparansulfaat proteoglycanen. Deze kennis kan gebruikt worden om specifieke remmers te ontwikkelen voor de verschillende vormen van u-PA waardoor de therapeutische toepassing van u-PA kan worden verbeterd.

Abbreviations

α_2-MR	α_2-macroglobulin receptor
α_2-M-T	α_2-macroglobulin activated with trypsin
apoE	apolipoprotein E
ASGPr	asialoglycoprotein receptor
ASOR	asialo-orosomucoid (asialo-α_1-acid glycoprotein)
BSA	bovine serum albumin
delta125-rscu-PA	rscu-PA lacking residue 10-135
D-Gal	D-galactose
EC	endothelial liver cell
GalNAc	N-acetyl-α-D-galactosamine
GGACK	Glu-Gly-Arg chloromethyl ketone
GlcNAc	N-acetyl-α-D-glucosamine
gp330	glycoprotein 330
GPI	glycosyl phosphatidyl inositol
GST	glutathione S-transferase
HMW-scu-PA	high molecular weight scu-PA
HMW-tcu-PA	high molecular weight tcu-PA
HSPGs	heparan sulfate proteoglycans
IgG	immunoglobulin G
KC	Kupffer cell
K_d	dissociation constant
kDa	kilo Dalton
LDL	low density lipoprotein
LDLr	low density lipoprotein receptor
LMW-tcu-PA	low molecular weight tcu-PA
LRP	low density lipoprotein receptor-related protein
NgF	N-glycosidase F
PAI-1	plasminogen activator inhibitor type 1
PBS	phosphate buffered saline
PC	parenchymal liver cell
PI-PLC	phosphatidylinositol phospholipase C
RAP	receptor-associated protein
RHL	rat hepatic lectin
rscu-PA	recombinant scu-PA
scu-PA	single-chain u-PA
tcu-PA	two-chain u-PA
t-PA	tissue-type plasminogen activator
u-PA	urokinase-type plasminogen activator
uPAR	u-PA receptor
ß-VLDL	ß-migrating very-low density lipoprotein
VLDLr	very-low density lipoprotein receptor
WFA	Wisteria floribunda agglutinin

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Curriculum Vitae

Marie Elisabeth van der Kaaden werd op 2 maart 1967 geboren te Berkel en Rodenrijs. In 1986 werd het diploma V.W.O. behaald aan de Christelijke Scholengemeenschap Melanchthon te Rotterdam. In datzelfde jaar werd begonnen met de studie Hoger Laboratorium Onderwijs tot chemisch analiste aan het Van 't Hoff Instituut Rotterdam/Delft. Na gekozen te hebben voor de specialisatie richting Biochemie werd een stage uitgevoerd bij de vakgroep Immunologie van de afdeling Kindergeneeskunde van het Academisch Ziekenhuis Leiden (E.M. Jol-van der Zijde, dr. M.J.D. van Tol). In juni 1990 werd de opleiding tot biochemisch analiste voltooid (*cum laude*). In datzelfde jaar werd een aanvang gemaakt met de studie Scheikunde aan de Rijksuniversiteit Leiden. In augustus 1992 werd het doctoraalexamen diploma behaald met als hoofdvak Moleculaire Genetica (prof.dr. P. van de Putte, dr. J.A. Brandsma).

Van oktober 1992 tot oktober 1996 was zij werkzaam als assistent in opleiding bij de sektie Biofarmacie van het Leiden/Amsterdam Center for Drug Research en het Gaubius Instituut, TNO Preventie en Gezondheid, alwaar onder leiding van prof.dr. Th.J.C. van Berkel, dr. J. Kuiper en dr. D.C. Rijken het in dit proefschrift beschreven onderzoek werd uitgevoerd. Sinds mei 1997 is zij werkzaam als wetenschappelijk medewerker bij IntroGene.

Nawoord

Met dit proefschrift wordt een periode van ruim vier jaar onderzoek doen afgerond. Gedurende deze periode heb ik met veel plezier bij de sektie Biofarmacie gewerkt, waarbij de nauwe samenwerking met het Gaubius Instituut (TNO-PG) zeker niet vergeten mag worden. Promoveren doe je niet alleen en een aantal mensen hebben bijgedragen aan de tot standkoming van dit proefschrift. Enkele mensen wil ik op deze plaats met naam noemen.

Allereerst mijn (oud)collega's bij Biofarmacie, voor de zeer prettige werksfeer op het lab en de interesse voor mijn onderzoek, ondanks dat het weinig van doen had met lipidedeeltjes en targetting. Mijn kamergenoten Patrick Rensen, Erik Rump en Kees Fluiter wil ik noemen voor de gezellige sfeer in onze kamer, waar naast tijd voor een praatje ook goed gewerkt kon worden. Het type-werk is nu echt af, dus de computer is weer voor jullie guys! Kar Kruijt wil ik noemen als onmisbare vraagbaak en hulp bij alle lab problemen. Vooral zijn hulp bij het verdoven van bijtgrage muizen en bij het verkrijgen van grote hoeveelheden rattelevers voor receptorisolatie heb ik zeer gewaardeerd. Leuk en leerzaam was de stageperiode van Lilian Martosoewondo. Jouw werk was een goede basis voor het PAI-verhaal, veel succes in Suriname.

De samenwerking met het één bruggetje verderop gelegen TNO-Gaubius heb ik als zeer plezierig ervaren. Natuurlijk mag Noor Groeneveld op deze plaats niet ontbreken, die gedurende de eerste jaren van mijn onderzoek voor accurate technische ondersteuning op het Gaubius heeft gezorgd. Naast het maken van allerlei u-PA brokken, heeft de stank van ontdooide levertjes je er niet van weerhouden om de interactie van u-PA met de door jou geisoleerde asialoglycoproteinereceptor aan te tonen. Alle andere collega's van het Gaubius wil ik noemen voor hun interesse, advies en gezelligheid tijdens congresbezoeken. Met name Marrie Barret-Bergshoef wil ik noemen voor het afronden van de WFA-experimenten gedurende het afgelopen jaar en Marjan Bekkers voor het leren van de aorta-shunt operatie en de Elisa's.

De plezierige en stimulerende samenwerking met de "Amsterdammers" Ivo Horn en Anton-Jan (Daan) van Zonneveld heeft bijgedragen aan het snel verstrijken van de laatste paar maanden promotietijd. Zonder het krijgen van voldoende PAI-1 en PAI-1-mutanten was hoofdstuk 7 nooit tot stand gekomen.

Verder wil ik mijn paranimfen Antoinette van Keulen en Saskia van der Kaaden noemen. Ik ben heel blij dat jullie me (Sas ondanks het feit geen voorstander te zijn van beestjesproeven) tijdens mijn promotie willen bijstaan. Daarnaast wil ik ook mijn (schoon)familie en vrienden noemen voor hun interesse in mijn onderzoek.

Mijn ouders wil ik speciaal noemen omdat zij mij instaat gesteld hebben en gestimuleerd hebben mijn studies te doorlopen, en altijd voor mij klaarstonden.

Tenslotte Richard, zonder jouw immer luisterend oor, steun en liefde zou het AIO-schap er voor mij heel anders hebben uitgezien.