The mannose receptor, localization and role in the clearance of tissue-type plasminogen activator.

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Cover illustration: modified picture of the mannose receptor expression in the liver, for original picture see page 152.

Stellingen behorende bij het proefschrift: The mannose receptor, localization and role in the clearance of tissue-type plasminogen activator.

1 De identiteit, de hoeveelheid en de onderlinge afstand van de eindstandige suikergroepen bepalen de affiniteit van een ligand voor de mannose receptor (dit proefschrift).

2 Het remmende effect van receptor liganden op de afbraak van weefseltype plasminogeenactivator (t-PA) door macrofagen *in vitro* is een goede indicatie voor het remmende effect van deze liganden op de afbraak van t-PA *in vivo* (dit proefschrift).

3 Het aantal receptoren op de celmembraan van de humane macrofaag dat in staat is om monoclonaal antilichaam 15-2 tegen de mannose receptor te binden weerspiegelt het activatietype van de macrofaag (dit proefschrift).

4 Als de variabiliteit in leverdoorbloeding bij patienten met een hartinfarct een rol speelt bij het risico op bloedingen bij thrombolytische therapie met t-PA (A. de Boer, proefschrift, Leiden 1990), dan kunnen stoffen die de interactie tussen t-PA en de mannose receptor blokkeren gebruikt worden om dit risico te reduceren (dit proefschrift).

5 De term "niet-specifieke afweer" is onjuist gezien de grote rol van specifieke suikerherkenning bij dit proces (Malhorta et al., Nature Medicine 1995; 1: 237-43, Prigozy et al. Immunity 1997; 6: 187-97, Jullien et al. J. Clin. Invest. 1997; 99: 2071-74).

6 Als immuunziektes zoals reumatoïde arthritis en de ziekte van Crohn veroorzaakt worden door langzaam delende bacteriën dan zouden deze ziektes niet behandeld moeten worden met middelen die de afweer onderdrukken (Rook et al. Immunology Today 1992; 13: 160-4).

7 De waarneming dat hersenschade vaker voorkomt bij (ex)professionele boksers dan bij (ex)amateurboksers (Jordan et al. JAMA 1997; 278: 136-140) roept de vraag op wat er eerder was, de hersenschade of de behoefte om professioneel te boksen.

8 De behoefte om genetische factoren vast te stellen die het risico op ongezondheid door slechte leefgewoontes vergroten, wordt bepaald door de wens om zoveel mogelijk slechte leefgewoontes in stand te houden.

9 Wanneer een placebo een genezende werking heeft, dient de rol van emoties bij de desbetreffende ziekte en/of het genezingsproces nader onderzocht te worden.

10 Het verschil tussen een gezond en een ziek persoon is dat alleen de zieke persoon zeker is van zijn toestand.

11 Aangezien de aarde het enige is wat ons allen bindt, zouden we de aarde niet moeten verdelen.

Leiden, 23 oktober 1997 **Femke Noorman**

Wherever there is mind, there is body,
and wherever body, mind

Hieronymus David Gaubius (1705-1780)

(citation from the Essay of 1747, De Regimine Mentis, a translation by L.J. Rather
annotated in Mind and Body in Eighteenth Century Medicine, University of California Press, 1965)

Aan mijn ouders
Ter nagedachtenis aan Noppes

CONTENTS

INTRODUCTION

The mannose receptor, localization and role in the clearance of tissue-type plasminogen activator.

The human body houses approximately 1 x 10^{13} cells. About 200 different cell types cooperate to create a balanced milieu in which they can survive. Cells create, control and maintain their surroundings by the use of molecular interactions. One way for the cells to maintain the environment is the production of enzymes and inhibitors. These proteins can modify the composition of extracellular structures and/or each other's action depending on their concentrations and localization.

The cells control the concentration of an extracellular protein by regulating its production and/or degradation. The degradation of some proteins can be controlled by the expression of endocytic receptors that specifically recognize and bind these molecules (ligands). After a molecule is bound to an endocytic receptor it is usually internalized and degraded by the cell. The localization of these ligands depends on the localization of the ligand-producing cells, the transport of the ligands through the body after release, and the localization of the cells that express the endocytic receptors.

A disease is caused by internal (e.g. genetic defects) and/or external (e.g. infection) factors that disturb the balanced environment. We can help the body to recover from a disease by the administration of drug(s). To anticipate the effects of a drug it is important to know its mechanisms of action and the concentrations that are required for these actions. The dosage, administration route, tissue distribution and degradation (clearance) of the drug determine the concentration of the drug at the site of action. Like endogenous ligands, some drugs are cleared by cells after binding to endocytic receptors.

This thesis describes the properties of the mannose receptor which is one of the endocytic receptors that mediate the clearance of an endogenous enzyme called tissue-type plasminogen activator (t-PA), which is also used as a drug.

The mannose receptor

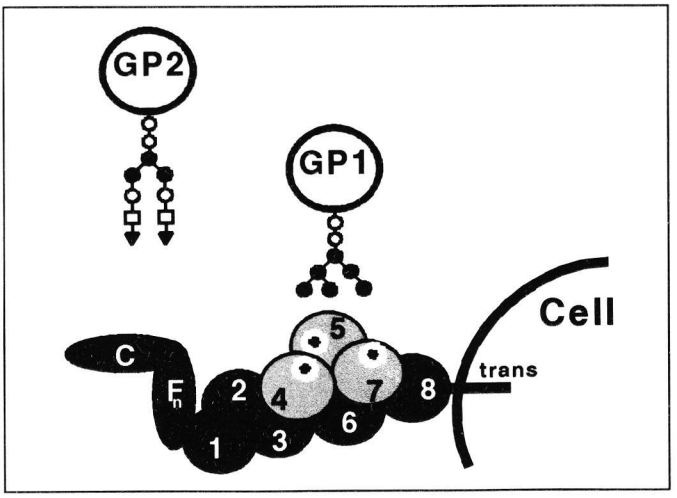
The mannose receptor is a 175 kDa type I transmembrane protein that belongs to the C-type lectin family. [1] The receptor consists of a N-terminal cysteine rich domain, a fibronectin type II domain, eight carbohydrate recognition domains (CRDs), a transmembrane domain, and a C-terminal cytoplasmatic tail [2] (Fig 1). The receptor has a low affinity for monosaccharides and a high affinity for ligands that bear poly-mannose structures. [3] The affinity of the ligand probably depends on the number of CRDs it can bind to. [4,5]

Fig 1

Schematic representation of the mannose receptor.

C: Cysteine rich domain, F_n: fibronectin type II repeat, eight carbohydrate recognition domains (1-8) with the mannose binding sites (● in 4,5 and 7), and a C-terminal transmembrane region with a cytoplasmatic tail).

GP1 represents a glycoprotein with a typical high mannose-type oligosaccharide that is able to bind to the mannose receptor. GP2 represents a glycoprotein with a typical complex-type oligosaccharide that is not able to bind to the mannose receptor. (O = N-acetyl-glucosamine, ● = mannose, □ = galactose, and ▼ = neuraminic acid) .



The mannose receptor mediates the endocytosis of glycoproteins bearing high mannose-type oligosaccharides such as lysosomal enzymes [6] and t-PA [7] (Fig 2) and the phagocytosis of pathogens carrying poly-mannose structures on their membrane. [8] After binding the ligand-receptor complex is internalized. Inside the cell the ligand is released in the acid environment of the endosome, the ligand is degraded in the lysosome while the receptor is recycled to the membrane. [9,10]

The 175 kDa mannose receptor has been isolated [11-13] from human macrophages, liver and placental tissue. Many cell types such as macrophages, [9] liver and splenic sinusoidal endothelial cells, [14,15] sperm cells, [16] airway smooth muscle cells, [17] lymphocytes and Langerhans cells [18] have been shown to bind mannose receptor ligands, but they may express other mannose binding proteins or mannose receptor-related proteins. [19-23]

By use of ligand inhibition studies the 175 kDa mannose receptor has been implicated in the mediation of several processes. In this way it has been shown that the mannose receptor expressed in the liver plays an important role in the clearance of its ligands from the circulation. [24] The mannose receptor also appears to play an important role in the innate immune system by mediating complement-independent uptake of pathogens [8] and in the adaptive immune system by the uptake of antigens prior to antigen presentation. [25,26] Furthermore the mannose receptor may play a role in lymphocyte homing to the spleen, [15] sperm fertility, [16] smooth muscle cell proliferation, [17] and fusion of macrophages into foreign body-type giant cells. [27]

Coagulation and fibrinolysis

After damage to a blood vessel the blood coagulates and a clot is formed, and after tissue repair the clot is degraded. Two processes are involved: coagulation and fibrinolysis. Both processes are cascades of enzymatic reactions; only a few enzymes need to be activated which in turn activate a lot of other enzymes. The coagulation cascade produces thrombin that converts soluble fibrinogen into an insoluble network of fibrin, a main component of a blood clot. The fibrinolytic cascade leads to the production of plasmin that converts fibrin into soluble fibrin degradation products, which results in the dissolution of the blood clot. Coagulation and fibrinolysis are controlled by production and degradation of enzymes and inhibitors. The balance of these processes determines whether the blood clot is formed or degraded. [28]

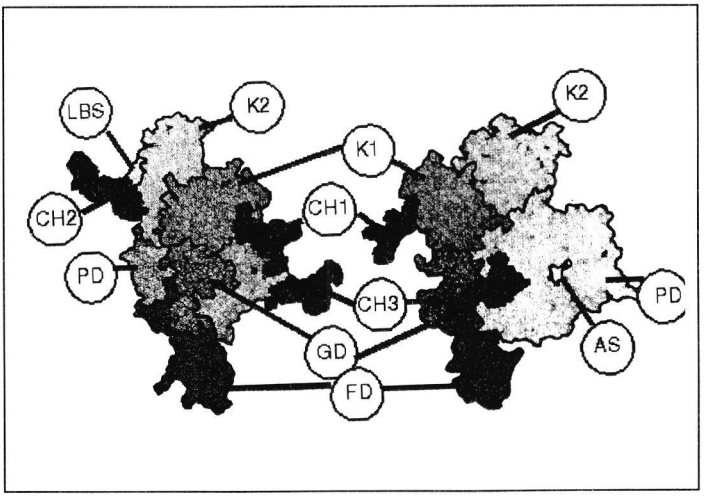
Tissue-type plasminogen activator (t-PA)

One of the molecules able to initiate the lysis of the blood clot is t-PA which converts plasminogen into plasmin. t-PA is a 70 kDa serine protease containing five domains; the finger domain, the growth factor domain, kringle 1, kringle 2, and the protease domain. The protein contains three N-linked glycosylation sites [29,30] (Fig 2). It contains one high mannose-type oligosaccharide on kringle 1 and one complex-type oligosaccharide on the protease domain. Kringle 2 may (type I t-PA) or may not (type II t-PA) contain a complex-type oligosaccharide. Furthermore t-PA is fucosylated in the growth factor domain.

Fig 2

Schematic representation of tissue-type plasminogen activator (t-PA).

Two views of a model of t-PA showing a hypothetical spatial arrangement of the domains. The finger domain (FD), growth factor domain (GD), Kringle 1 (K1) which is glycosylated with a high mannose-type oligosaccharide (CH1), Kringle 2 (K2) which contains the lysine binding site (LBS) and a complex-type oligosaccharide (CH2) and the protease domain (PD) which contains the active site (AS) and a complex-type oligosaccharide (CH3) (modified from Opdenakker et al. [29]).



In a healthy situation t-PA circulates in low concentrations in the blood. Synthesis and blood clearance of t-PA as well as inhibition of t-PA by plasminogen activator inhibitor-1 (PAI-1) control the activity of t-PA in blood. The t-PA in the blood is continuously produced by the endothelial cells lining the blood vessels, [31] and continuously cleared mainly by the liver. [32,33] The clearance of t-PA is very rapid. Within 5 minutes half of an injected dose of t-PA has disappeared from the blood. The clearance of t-PA is a receptor-mediated process. The mannose receptor is one of the endocytic receptors expressed in the liver that mediate the clearance of t-PA. [7] By use of recombinant technology, t-PA can be made outside the body. Recombinant t-PA is successfully used as a drug in thrombolytic therapy [34] and may be useful in antithrombotic therapy. [35]

Aim of this thesis

The thrombolytic and antithrombotic effect of t-PA depend on its concentration in the blood. The mannose receptor is one of the receptors that mediates the clearance of t-PA from the blood. We hypothesized that by blocking the binding of t-PA to this receptor it might be possible to decrease the clearance of t-PA and thereby increase the efficacy of both endogenous and exogenous t-PA. Inhibitors of the t-PA-mannose receptor interaction may thus be useful drugs in thrombolytic and antithrombotic therapy. The aim of this study was to extend our knowledge of the mannose receptor in order to develop efficient mannose receptor inhibitors. These inhibitors may in the future be used to prevent or to treat thrombotic (and other) diseases.

To study the human mannose receptor-t-PA interaction and to evaluate the efficacy of inhibitors we developed and characterized two *in vitro* assays (chapter 1 and 2). Monoclonal antibodies against the human mannose receptor were developed as a tool to specifically study the 175 kDa mannose receptor that is able to bind t-PA (chapter 3). To assess the possible role(s) of the mannose receptor in man these monoclonal antibodies were used to evaluate the mannose receptor expression on cells *in vitro* and in human tissues under physiological and pathological conditions (chapter 4,5). A panel of mannose receptor inhibitors was synthesized and tested for their efficacy to inhibit the interaction of t-PA with the mannose receptor *in vitro* and the t-PA clearance *in vivo* (chapter 6,7). To provide evidence for a possible antithrombotic effect of mannose receptor inhibitors in man, we studied the effect of the antithrombotic drug, dextran, on the t-PA-mannose receptor interaction *in vitro* and its effect on exogenous t-PA clearance and on endogenous t-PA concentrations *in vivo* (chapter 8). The results of this thesis are discussed in the context of the literature concerning t-PA, the mannose receptor and other t-PA clearance receptors (chapter 9).

The main outcomes of this thesis are that the mannose receptor is expressed by few human cell types. The expression of the mannose receptor on macrophages is highly regulated and depends on the type of macrophage activation. The functioning of the mannose receptor in the liver influences t-PA plasma concentrations. Mannose receptor inhibitors inhibit t-PA plasma clearance and thereby increase t-PA plasma concentrations. Thus mannose receptor inhibitors can be considered as a new strategy to increase the t-PA concentration in blood and thereby increase the efficacy of thrombolytic and antithrombotic therapy.

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CHAPTER 1

Role of carbohydrate in the binding of tissue-type plasminogen activator to the human mannose receptor.

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SUMMARY

The 175 kDa mannose receptor is one of the receptors that mediates the clearance of tissue-type plasminogen activator (t-PA). The affinity of t-PA for the mannose receptor is much higher than the affinity of other high mannose-type oligosaccharide-containing glycoproteins. In order to find an explanation for this high affinity we studied the biochemical interaction of various forms of t-PA with the isolated human mannose receptor in several *in vitro* binding assays.

t-PA showed a high affinity (Ki = 0.2 nM) for the mannose receptor and the interaction could be fully inhibited by mannan or polyclonal antibodies against the mannose receptor. The interaction was not affected by non-glycosylated t-PA. The high affinity differed slightly between t-PAs synthesized by various cell types and between various glycoforms of t-PA. No statistically significant difference in affinity between t-PA and t-PA complexed to inhibitors was observed. In contrast to intact t-PA, a trypsin digest of t-PA had a low affinity (Ki = 0.5 μM) for the mannose receptor. Both intact and trypsin digests of the high mannose-type oligosaccharide-containing glycoproteins ribonuclease B and ovalbumin had a low affinity (Ki 0.5 - 1.5 μM) for the mannose receptor.

We conclude that neither protein-protein interactions, nor the complex-type oligosaccharides and the fucose residue on t-PA contribute significantly to the high affinity binding of t-PA. We suggest that the conformation of the high mannose-type oligosaccharide on t-PA is influenced by the protein moiety of t-PA in such a way that the oligosaccharide has a high affinity for the mannose receptor.

INTRODUCTION

The mannose receptor was originally found to be expressed on alveolar macrophages [1] and the 175 kDa protein has been isolated from macrophages,[2] placenta[3] and liver.[4] The mannose receptor expressed on macrophages has been implicated as mediating different processes such as endocytosis of glycoproteins,[1] phagocytosis of organisms having poly-mannose structures on their membrane,[5] antigen uptake[6,7] and macrophage fusion into foreign body-type giant cells.[8] The mannose receptor expressed in the liver has been shown to mediate rapid clearance of high mannose-type oligosaccharide containing glycoproteins from the circulation such as tissue-type plasminogen activator (t-PA).[9] t-PA is a serine protease that activates fibrinolysis by converting plasminogen into plasmin, which cleaves fibrin into soluble degradation products.[10] t-PA is successfully used for thrombolytic therapy, for instance after myocardial infarction.[11] Because of its rapid clearance high doses of t-PA are required to obtain thrombolysis. Thus inhibition of mannose receptor-mediated clearance could be useful in increasing the efficacy of t-PA in thrombolytic therapy.[12]

It has been shown that the binding of t-PA to the mannose receptor is mediated probably by its high mannose-type oligosaccharide.[13,14] The binding is pH and Ca^{2+} dependent and can be inhibited by saccharides such as mannose and mannosylated albumin.[4,14] The affinity of t-PA for the mannose receptor (Kd 1-4 nM[4,13-15]) is much higher than the affinity of other high mannose-type oligosaccharide containing glycoproteins such as ribonuclease B, ß-glucuronidase and ovalbumin (Kd 60-600 nM[15-18]). It is not known which structural elements of t-PA are responsible for this higher affinity. Furthermore the clearance of t-PA complexed to plasminogen activator inhibitor type 1 (PAI-1) appears to be slower in human subjects.[19,20] This might be caused by a lower affinity of t-PA-PAI-1 for the mannose receptor. The affinity of t-PA-proteinase inhibitor complexes for the mannose receptor is, however, not known.

We initiated this study to determine which structures of t-PA are responsible for the high affinity. We isolated the 175 kDa mannose receptor from human placenta and developed several *in vitro* binding assays. In these assays we studied the biochemical interactions of various forms of t-PA and t-PA-proteinase inhibitor complexes with the mannose receptor. We show that the interaction of t-PA with the mannose receptor varied slightly between the various t-PA preparations, and differed strongly from other high mannose-type oligosaccharide containing glycoproteins, probably by an effect of the protein moiety of t-PA on the conformation of the oligosaccharide.

MATERIALS AND METHODS

Materials

The human mannose receptor was isolated and purified from placental tissue on a mannosylated-albumin-Sepharose column. [4,14] The column was eluted with 0.2 M α-D-mannose, first in a Triton X-100 free buffer and then in a 1% (v/v) Triton X-100 containing buffer. The isolated protein was demonstrated to be a single 175 kDa protein in SDS-polyacrylamide gel electophoresis. [4] Polyclonal antibodies were raised against the purified 175 kDa human mannose receptor in a goat and IgG's were isolated from the antiserum by sodium sulphate precipitation. [21] Control IgG's were isolated similarly from nonimmune goat serum. Melanoma t-PA (mt-PA) was purified from a recombinant human melanoma cell culture. [22] Recombinant t-PA from CHO cells (rt-PA) was Actilyse (Boehringer Ingelheim, Ingelheim, Germany). Recombinant non-glycosylated t-PA produced in *E. coli* (BM 06.021, molecular mass 59 kDa [23]) was a generous gift from Boehringer Mannheim (Mannheim, Germany). A T60A t-PA mutant, lacking the O-linked fucose residue in the growth factor domain, as well as a control t-PA preparation produced similarly were kindly donated by Dr. J. Henkin (Abbott Laboratories, Abbott Park, Il., USA). Two-chain rt-PA was prepared by treating single chain rt-PA (Actilyse) with plasmin [24] and by inhibiting residual plasmin activity with 5 μM D-Val-Phe-Lys-chloromethyl ketone (Calbiochem, La Jolla, CA, USA). Type I and type II mt-PA were separated by using lysine-Sepharose chromatography, as described before. [25] Melanoma t-PA was biotinylated as described earlier. [26] Mannan (a poly-mannose structure isolated from *Saccharomyces cerevisiae*) was obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Mannose receptor binding assays

Triton X-100 free purified mannose receptor (0.15 μg/ml) in 20 mM Tris-HCl buffer, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.4 (coating buffer) was adsorbed overnight at 4 °C to the wells of polyvinyl chloride microtitre plates (Flow Laboratories, Irvine, UK). After treating the wells with binding buffer (coating buffer supplemented with 0.5% (v/v) Tween 80 and 1 mg/ml BSA) for 0.5 hour at room temperature, the wells were incubated for 2 hours at room temperature with increasing concentrations of biotinylated mt-PA or biotinylated mt-PA-proteinase inhibitor complexes in binding buffer. Bound mt-PA was quantified by incubation of the wells at room temperature for 1 hour with alkaline phosphatase-conjugated streptavidine (Amersham, Buckinghamshire, UK). After washing, the wells were incubated at 25 °C with the chromogenic substrate p-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, MO, USA). The results were corrected for non-specific binding determined in wells which were "coated" with buffer without mannose receptor. Competitive binding studies were performed by incubating immobilized mannose receptor with mixtures of a non-saturating concentration of biotinylated mt-PA (1-3 nM) and increasing concentrations of inhibitors. When only limited amounts of ligands were available, the binding assay was modified as follows. The immobilized mannose receptor was incubated with 0.14 nM unlabelled t-PA preparations. The wells were washed twice with binding buffer and once with 0.1 M Tris-HCl buffer, 5 mM $CaCl_2$, 0.1% (v/v) Tween 80, pH 7.4. Bound t-PA was eluted with the latter buffer in which $CaCl_2$ was replaced by 10 mM EDTA and then quantified by the t-PA activity assay of Verheijen et al. [27]

Preparation and quantification of biotinylated t-PA inhibitor complexes

Melanoma t-PA-PAI-1 complex was prepared as described before. [28] Melanoma t-PA-α_2-antiplasmin complex was prepared by incubating 1 μM mt-PA in 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 100 mM 6-aminohexanoic acid (6-AHA) and 0.01% (v/v) Tween 80 with 2.2 μM α_2-antiplasmin (Biopool, Umeå, Sweden) for 21 hrs at 37 °C. Control mt-PA was incubated similarly without α_2-antiplasmin. The three preparations were biotinylated [26] and gel filtered on a Sephacryl S200 HR (Pharmacia, Uppsala, Sweden) column of 100 x 1 cm in 50 mM Tris-HCl, pH 7.5, containing 1.6 M KSCN and 0.001% (v/v) Tween 80 to remove traces of free mt-PA. The protein preparations were supplemented with BSA (1 mg/ml final concentration), dialyzed against binding buffer of the mannose receptor binding assay, and quantified with an ELISA for t-PA (Biopool, Umeå Sweden) using biotinylated mt-PA as a standard. The ELISA measured t-PA-inhibitor complexes as efficiently as free t-PA.

Preparation of trypsin digests of glycoproteins

The glycoproteins rt-PA, ribonuclease B (Sigma, Chemical Co., St Louis, MO, USA) and ovalbumin (Fluka, Buchs, Switzerland) and control buffer, were treated with trypsin (Serva, Heidelberg, Germany) essentially as described for ovalbumin by Glabe et al. [29] Residual trypsin activity was blocked with 10 KIU/ml aprotinin (Bayer, Leverkusen, Germany). SDS-polyacrylamide gel electrophoresis on 18% (w/v) gels showed that there was no intact glycoprotein left in the solution.

Statistics

The sigmoidal binding and inhibition curves were analysed using nonlinear regression analysis with the computer program GraphPAD (ISI Software, Philadelphia, PA, USA), yielding the dissociation constant Kd or half maximal inhibitory concentration (IC_{50}). The inhibitory constant obtained in the inhibition curve, Ki, was calculated from the IC_{50}, the ligand concentration and the ligand dissociation constant by using the Michaelis-Menten equation Ki = IC_{50}/(1+ ligand concentration/Kd ligand). "Goodness of fit" was assessed by evaluating the actual distance of the measurements from the fitted line (no weighing). Data are presented as mean ± standard deviation. Significance of differences was assessed by use of the non-parametric Mann Whitney test: two-tailed $P < 0.05$ is defined as significantly different.

RESULTS

The affinity of melanoma t-PA (mt-PA) for the isolated mannose receptor was determined in two different manners. Increasing concentrations of biotinylated mt-PA were added to the immobilised receptor, which resulted in a typical binding curve (Fig 1A). Alternatively, a non-saturating concentration of biotinylated mt-PA was added to the receptor in the presence of increasing concentrations of unlabelled mt-PA, which resulted in a typical inhibition curve (Fig 1B). On average the dissociation constant determined in the binding assay (Kd) was 2.4 ± 0.8 nM (mean ± SD, n=6) and the inhibitory constant determined in the inhibition assay (Ki) was 0.23 ± 0.11 nM (n=8). The lower affinity obtained in the binding assay was probably due to the biotinylation of t-PA.

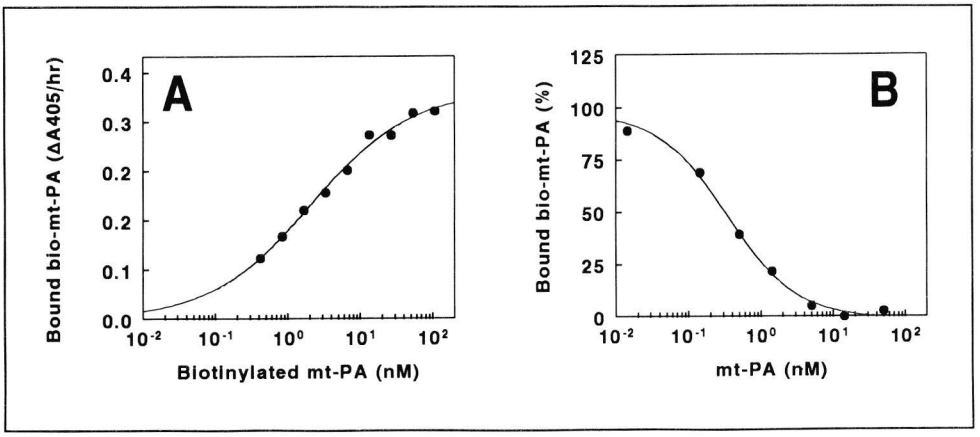


Fig 1 Affinity of mt-PA in the mannose receptor binding assays.

A: typical binding curve of biotinylated mt-PA (bio-mt-PA). Multiwells, coated with isolated human mannose receptor, were incubated with increasing concentrations of bio-mt-PA. After incubation, ligand binding was determined. Binding of bio-mt-PA was corrected for aspecific binding determined in uncoated wells and plotted as ΔA405/hr. B: typical inhibition curve of mt-PA. Multiwells, coated with isolated human mannose receptor, were incubated with a non-saturating concentration of bio-mt-PA in the presence of increasing concentrations unlabelled mt-PA. After incubation, bio-mt-PA binding was determined and corrected for aspecific binding determined in uncoated wells. Binding of bio-mt-PA was expressed as a percentage of the control (without unlabelled mt-PA).

The specificity of mt-PA binding to the immobilized mannose receptor is shown in Fig 2. Mannan, a known ligand of the mannose receptor inhibited mt-PA binding with a Ki value of 0.14 µg/ml, while non-glycosylated t-PA synthesized by *E. coli* did not inhibit biotinylated mt-PA binding (Fig 2A). In addition goat polyclonal antibodies raised against the human mannose receptor were able to completely inhibit the binding of mt-PA to the mannose receptor, whereas non-immune immunoglobulines had no effect (Fig 2B). Since the binding of t-PA to the mannose receptor was fully inhibited by mannan and not inhibited by non-glycosylated t-PA, it is unlikely that protein-protein interactions contribute significantly to the high affinity binding of t-PA. Furthermore the high affinity of t-PA for the mannose receptor in the binding assay did not involve lysine binding sites of t-PA or clustered t-PA molecules, since the lysine analog 6-aminohexanoic acid (6-AHA), which fully dissolves t-PA, did not inhibit the binding of t-PA to the mannose receptor (6-AHA concentration range 0.02 - 200 mM, results not shown).

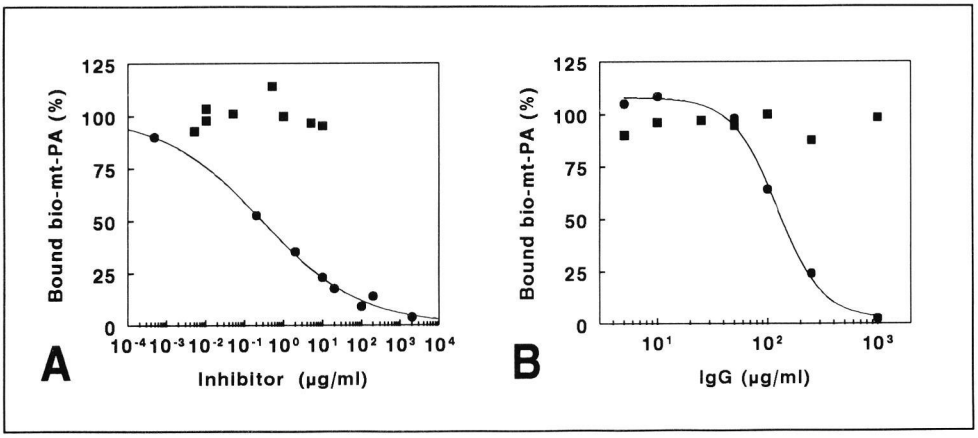


Fig 2 **Specificity of mt-PA binding in the mannose receptor binding assay.**
The same procedure as described in the legend of Fig 1B was followed, in this case increasing concentrations mannan (●) or non-glycosylated t-PA (■) were used as inhibitor in Fig 2A. Anti-mannose receptor polyclonal antibodies (●) or non-immune antibodies (■) were used as inhibitor in Fig 2B.

The above-mentioned experiments were performed with t-PA synthesized by melanoma cells (mt-PA). Recombinant t-PA synthesized by CHO cells (rt-PA) might have a slightly different glycosylation pattern from mt-PA. In the inhibition assay, the affinity of rt-PA for the mannose receptor was also high (Ki = 0.70 ± 0.26 nM, n=8), but about three times lower than that of mt-PA (K_i = 0.23 ± 0.11 nM, n=8, $P<0.005$).

The rt-PA preparation, consisting largely of the single-chain form, was treated with plasmin and converted to the two-chain form. [24] Fig 3 shows that the two forms of rt-PA had equal affinities for the mannose receptor. These results indicate that the high affinity of t-PA is not dependent on the conformation of the protease domain of t-PA.

To determine differences in affinity of ligands only available in low amounts a modified binding assay was used. A low concentration (1.3 nM) of unlabelled t-PA was incubated with the immobilized mannose receptor. After washing the bound t-PA was eluted from the wells by the use of EDTA and the activity of the eluted t-PA was determined. As shown in Fig 4 similar amounts of type I and type II mt-PA bound to the mannose receptor which indicated that there was also no difference in affinity between type I and type II mt-PA. Both glycoforms of t-PA contain the high mannose-type oligosaccharide on kringle 1 and the complex-type oligosaccharide on the protease domain. Type I t-PA contains a complex-type oligosaccharide on kringle 2, whereas type II t-PA lacks this oligosaccharide. [25,30-33] These results indicate that the complex-type oligosaccharide on kringle 2 is not involved in the high affinity of t-PA for the mannose receptor.

As expected from the different affinities determined in the above-mentioned experiments less rt-PA than mt-PA type I or type II bound to the mannose receptor (Fig 4). In this modified binding assay, we also tested the affinity of a t-PA mutant, T60A, that lacks the O-linked fucose residue in the growth factor domain. Control t-PA, synthesized by the same cell type as the mutant t-PA, had a similar affinity as rt-PA since similar percentages bound to the mannose receptor. Slightly less mutant t-PA than control t-PA (2.4 ± 0.3% versus 3.6 ± 0.1%, respectively, $P<0.05$) bound to the mannose receptor which indicated that the mutant t-PA had a slightly lower affinity for the mannose receptor. Since the amount of mutant t-PA that did bind to the mannose receptor at these low concentrations was still high it is unlikely that the fucose unit strongly contributes to the high affinity of t-PA for the mannose receptor.

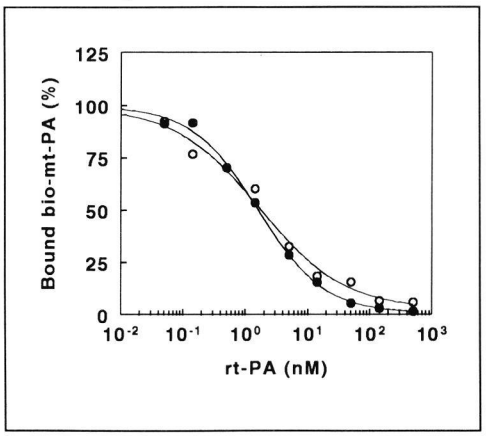


Fig 3 **Affinity of single-chain and two-chain rt-PA in the mannose receptor binding assay.**
The same procedure as described in the legend of Fig 1B was followed. In this case increasing concentrations of single-chain (●) or two-chain (○) rt-PA were used as inhibitor.

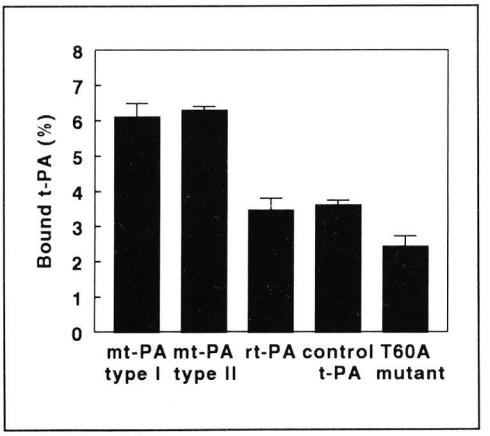


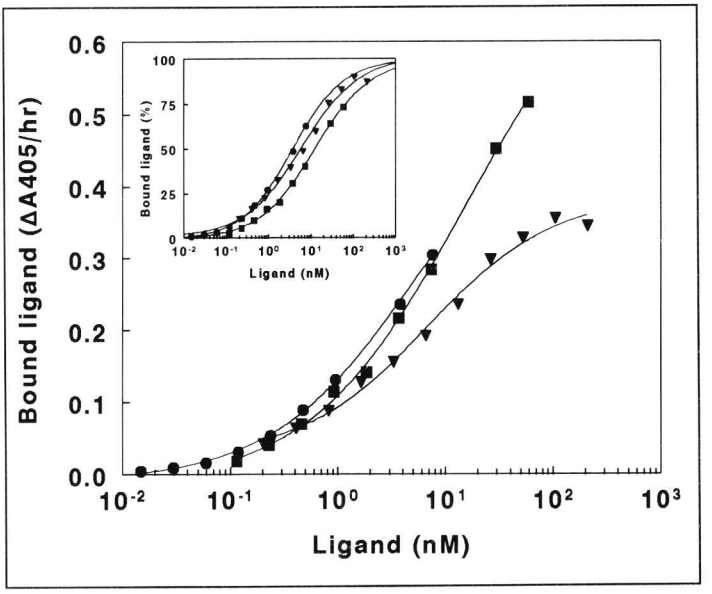
Fig 4 **Affinity of type I mt-PA, type II mt-PA, rt-PA, mutant control t-PA and the fucose lacking mutant T60A for the immobilized mannose receptor.**
Multiwells, coated with isolated human mannose receptor, were incubated with 0.14 nM unlabeled t-PA variants. After incubation, bound t-PA was eluted using EDTA and t-PA activity was measured. Binding of t-PA was expressed as percentage of the added activity of t-PA (n= 3 to 6).

To determine whether complexation of t-PA to plasma proteinase inhibitors would affect the affinity of t-PA for binding to the mannose receptor, mt-PA and mt-PA-inhibitor complexes were biotinylated and binding curves were generated. As shown in Fig 5, mt-PA complexed to proteinase inhibitors still binds to the mannose receptor with high affinity. There was no statistically significant difference in affinity between mt-PA and mt-PA complexed to α_2-antiplasmin or PAI-1 (Fig 5, inset).

Fig 5

Affinity of mt-PA-proteinase inhibitor complexes in the mannose receptor binding assay.

Melanoma t-PA and mt-PA complexed to PAI-1 or α_2-antiplasmin were biotinylated and the affinity of these ligands were determined in the binding assay as described in the legend of Fig 1A. mt-PA (▼), mt-PA-PAI-1 (●), mt-PA-α_2-antiplasmin (■). The inset shows the non-linear regression curves when the binding of the ligand is expressed as a percentage of the maximal binding of the ligand. These curves represent a correction for the differences in biotinylation of the ligands.



Using the competitive inhibition assay, we compared the affinity of t-PA to those of two other high mannose-type oligosaccharide containing ligands, ribonuclease B and ovalbumin. As shown in Fig 6A, rt-PA had a much higher affinity (Ki = 1.0 nM) than ribonuclease B and ovalbumin (Ki = 505 and 549 nM, respectively) for binding to the isolated mannose receptor. To determine whether the protein structures of these ligands would be responsible for the observed differences in binding affinity, trypsin digests were made of above-mentioned ligands. The trypsin digest of rt-PA had an approximately 500-fold lower affinity for the mannose receptor than intact rt-PA (Fig 6B), whereas the trypsin digests of ovalbumin and ribonuclease B only had a 2-3 times lower affinity for the mannose receptor than the intact glycoproteins. After trypsin digestion, the affinity of the degraded glycoproteins for the mannose receptor was similar for all three glycoproteins. Thus the protein structure of t-PA appeared to be involved in the high affinity of the high mannose-type oligosaccharide of t-PA for binding to the mannose receptor.

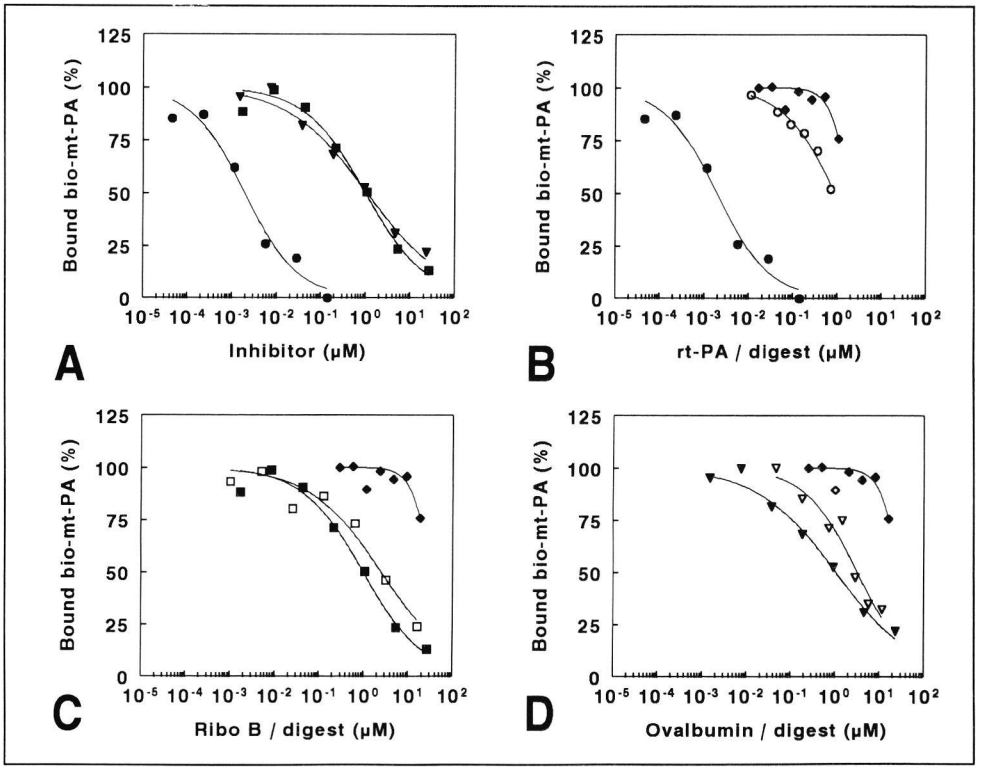


Fig 6 **Affinity of t-PA, ribonuclease B, ovalbumin and their trypsin digests for the immobilized mannose receptor.**
The same procedure as described in the legend of Fig 1B was followed. In this case increasing concentrations of intact glycoproteins or trypsin digests were used. A: inhibition curves of rt-PA (●), ribonuclease B (■), and ovalbumin (▼). The inhibition curves of the intact ligands were compared to the inhibition curves of their trypsin digest (open symbols) and of control buffer (trypsin treated buffer without glycoprotein, ◆) in B-D. The 'concentration' of the control buffer represents the amount of trypsin present in the trypsin digest shown in the figure. B: intact t-PA, t-PA digest and control buffer, C: intact ribonuclease B, ribonuclease B digest and control buffer, D: intact ovalbumin, ovalbumin digest and control buffer.

DISCUSSION

Previous studies in our laboratory showed that t-PA probably binds with its high mannose-type oligosaccharide in a Ca^{2+} dependent way to the mannose receptor. The binding of t-PA can be inhibited by mannose, fucose, N-acetylglucosamine and synthetic poly-mannose ligands.[4,14,15] The affinity of t-PA for the mannose receptor is much higher than the affinity of other high mannose-type oligosaccharide containing glycoproteins.[15-18] This study was initiated to determine which structures of t-PA are responsible for this higher affinity.

We isolated the human mannose receptor from placenta and developed several *in vitro* assays, in which the mannose receptor was immobilized. By the use of binding and competitive inhibition assays it was shown that the t-PA affinity of binding to the isolated mannose receptor had a high affinity (0.23 - 2.4 nM). Similar high affinities were reported for the binding of t-PA to the mannose receptor isolated from bovine alveolar macrophages[14] and the mannose receptor expressed by rat liver endothelial cells.[13]

The specificity of the binding of t-PA to the mannose receptor immobilized on mictrotitre plates was demonstrated clearly as mannan and polyclonal anti-mannose receptor antibodies were able to inhibit t-PA binding completely. We investigated if oligosaccharides other than the high mannose-type oligosaccharide on kringle 1 were involved in the high affinity binding of t-PA. The carbohydrate structures on mt-PA and rt-PA have been determined in various studies. [30-34] The complex-type oligosaccharides differed in the linkage of the terminal sialic acid on the galactose (α2-6 linked in mt-PA and α2-3 linked in rt-PA), [32] the presence of a biantennary complex-type oligosaccharide with a terminal N-acetylgalactosamine (GalNAc) structure in mt-PA, [34] and the presence of terminal intersecting N-acetylglucosamine (GlcNAc) residues in rt-PA. [32] It has been shown that terminal intersecting GlcNAc residues can influence the orientation of the oligosaccharide on the protein. [35] Alternatively, t-PA binding to the mannose receptor might directly involve this GlcNAc. However it has also been shown that peptides containing complex-type oligosaccharides with or without terminal intersecting GlcNAc residues do not bind to the mannose receptor. [17] There proved to be a slight but significant difference in affinity of mt-PA and rt-PA for binding to the isolated mannose receptor. Though the affinity of rt-PA was lower than the affinity of mt-PA, the affinity of rt-PA was still very high. This indicates that the terminal intersecting GlcNAc residues of the complex-type oligosaccharides do not contribute to the high affinity of t-PA for the mannose receptor.

There was no difference in the affinity of type I and type II t-PA for the mannose receptor. This shows that the complex-type oligosaccharide on kringle 2 is not involved in the binding of t-PA to the mannose receptor. Substitution of the threonine that contains an O-linked fucose residue with a (non glycosylated) alanine in the growth factor domain, [36] slightly reduced the affinity of t-PA for the mannose receptor. Since the mutant t-PA did considerably bind to the mannose receptor at low concentrations it is however unlikely that the fucose unit is important for the high affinity of t-PA for the mannose receptor.

Complex formation of t-PA with its natural inhibitors PAI-1 or α_2-antiplasmin did not significantly affect the affinity for binding to the mannose receptor. Interaction of t-PA-PAI-1 with the mannose receptor explains the uptake of the complex by liver endothelial cells *in vivo*. [28] The results indicate that the protease domain involved in the complex formation with these inhibitors does not contribute to the binding of t-PA to the mannose receptor. It has been suggested that t-PA-PAI-1 complexes are cleared more slowly than free t-PA *in vivo* in man. [19,20] Since the other receptor that mediates t-PA clearance, the low density lipoprotein receptor-related protein (LRP), [12] appeared to have a higher affinity for t-PA-PAI-1 than for t-PA, [37] the apparently lower clearance rate of t-PA-PAI-1 in man cannot be explained by reduced affinity of the complex for the mannose receptor or the LRP.

The mannose receptor contains 8 carbohydrate recognition domains (CRD). [38] It has been shown that the mannose receptor has a high affinity for mannan (Ki = 100 nM). [39] Since mannan has a much lower affinity (Ki in the µM to mM range) for mutant mannose receptors lacking one or more of these CRDs it was concluded that CRD 4,5 and 7 are essential for the high affinity binding (Ki in the nM range) of mannan. [39,40] Various studies indicated that the distance between the terminal mannose residues of polymannosides or oligosaccharides determines the affinity of ligand for the mannose receptor. [15,41,42]

The affinities of ribonuclease B and ovalbumin (both high mannose-type oligosaccharide-containing glycoproteins) for the mannose receptor were found to be similar to the affinities reported for ribonuclease B, ovalbumin and ß-glucuronidase. [15-18] We showed that all t-PA variants studied had a much higher affinity for the mannose receptor than ribonuclease B and ovalbumin. Since the CRD 4,5 and 7 are essential for the high affinity to the receptor, we suggest that t-PA binds simultaneously to the mannose receptor CRD 4,5 and 7, which results in high affinity binding, whereas ovalbumin or ribonuclease B may only bind to two out of three CRDs which results in a much lower affinity. However, besides some microheterogenity, the structures of the high mannose-type oligosaccharides of t-PA, [30-34] ovalbumin [43] and ribonuclease B [44] are essentially the same, and we showed that the other oligosaccharides present on t-PA did not seem to be involved in the high affinity binding of t-PA.

It has been shown that the protein core of complement C3 and ribonuclease B restricts the binding of the lectins conglutinin and mannose binding protein to the oligosaccharide probably by influencing the conformation or the accessibility of the oligosaccharide. [45] Thus we hypothesized that the protein part of ribonuclease B and ovalbumin restricts the binding of the high mannose-type oligosaccharide to the mannose receptor. However, the trypsin digests of ribonuclease B and ovalbumin had a 2-3 times lower affinity than the intact glycoproteins. This showed that the lower affinity of ovalbumin and ribonuclease B did not originate from restriction by the protein core.

Trypsin digests of t-PA, ribonuclease B and ovalbumin all had a similar low affinity (Ki 0.5-1.5 µM). This showed that the high affinity of intact t-PA for the mannose receptor was not caused by a different type of high mannose-type oligosaccharide. Since non-glycosylated t-PA nor 6-AHA could inhibit the binding of t-PA to the mannose receptor, this interaction is not likely to be mediated by protein-protein interactions. The trypsin digests of t-PA had a 500 times lower affinity than intact t-PA. These findings indicate that the protein structure is involved in the high affinity of the high-mannose-type oligosaccharide of t-PA for binding to the mannose receptor.

It is possible that the protein structure of t-PA influences the conformation or accessibility of its high mannose-type oligosaccharide. For IgM and ribonuclease B it has been shown that the motion of the oligosaccharides is not influenced by the protein structures. [46] However, for the glycoprotein IgG it has been shown that the protein core can interact with the terminal saccharides of its oligosaccharide in such a way that the motion of the oligosaccharide is restricted. [47] More rigid oligosaccharides have been suggested to have a higher affinity for lectins. [48] Since trypsin digests of t-PA had a much lower affinity for the mannose receptor, we suggest that the protein core of t-PA interacts with the high mannose-type oligosaccharide in such a way that the oligosaccharide on the intact protein can interact with a higher affinity with the mannose receptor than the free oligosaccharide and the high mannose-type oligosaccharide on ribonuclease B or ovalbumin.

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CHAPTER 2

Degradation of tissue-type plasminogen activator by human monocyte-derived macrophages is mediated by the mannose receptor and by the low density lipoprotein receptor-related protein.

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SUMMARY

The balance of tissue-type plasminogen activator (t-PA) production and degradation determines its concentration in blood and tissues. Disturbance of this balance may result in either increased or decreased proteolysis. In the present study we identified the receptor systems involved in the degradation of t-PA by human monocytes/macrophages in culture.

Monocytes were cultured and became macrophages within 2 days. At 4 °C, ^{125}I-t-PA bound to macrophages with high (apparent dissociation constant (kd), 1 to 5 nmol/L) and low affinity (kd > 350 nmol/L). At 37°C, the cells internalized and degraded t-PA via the high affinity binding sites, which were partially inhibited by mannan. The low affinity binding sites were 6-aminohexanoic acid-inhibitable and not involved in t-PA degradation. Degradation of t-PA was upregulated during differentiation of monocytes to macrophages. Dexamethasone further upregulated the mannan-inhibitable t-PA degradation. Lipopolysaccharide downregulated both mannan-inhibitable and non-mannan-inhibitable t-PA degradation. Non-mannan-inhibitable degradation was completely blocked by recombinant 39-kD receptor-associated protein (RAP, inhibitor of lipoprotein receptor-related protein (LRP)), whereas mannan-inhibitable degradation was blocked by the addition of a monoclonal antibody against the mannose receptor. No differences between the degradation of t-PA and functionally inactivated t-PA were observed.

We conclude that human monocyte-derived macrophages are able to bind, internalize, and degrade t-PA. Degradation of t-PA does not require complex formation with plasminogen activator inhibitors. The macrophages use two independently regulated receptors, namely the mannose receptor and LRP, for the uptake and degradation of t-PA.

INTRODUCTION.

Tissue-type plasminogen activator (t-PA) is a serine protease that converts plasminogen into plasmin, which in turn converts fibrin into fibrin degradation products, and is synthesized and secreted mainly by vascular endothelial cells. Recombinant t-PA is at present used as a thrombolytic drug, for instance, after a myocardial infarction. This glycoprotein (70 kD) contains one high mannose-type oligosaccharide and one or two complex-type oligosaccharides. [1] It is rapidly cleared from the plasma probably by the mannose receptor on liver endothelial cells and the α_2-macroglobulin receptor/ low density lipoprotein receptor-related protein (LRP) on hepatocytes. [2-4]

Macrophages arise from stem cell precursors in the bone marrow, circulate as monocytes in blood, and migrate into tissues to assume their mature state. The functional state of the macrophage (see reviews [5-7]) is dependent on the tissue of maturation and on the (pathologic) conditions in the environment. Many stimuli induce macrophages to secrete plasminogen activators to stimulate plasmin-mediated proteolysis. The plasminogen activators may be of the urokinase-type (u-PA) [8,9] or tissue-type (t-PA). [10,11]

Monocytes are recruited to the site of inflammation, and within 3 days macrophages represent the major cell type at this site. [12] When these macrophages are eliminated (using anti-macrophage serum), clearance of fibrin, dead cells and other debris necessary for repair is delayed. [13] For this clearance it is essential that the macrophage has several ways to regulate its endocytotic activities and the proteolytic activities in its environment.

This study was designed to investigate whether human monocyte-derived macrophages are able to bind, internalize, and degrade t-PA. Because macrophages express the mannose receptor [14] and may recognize t-PA through its high mannose-type oligosaccharide, we specifically studied the potential role of this receptor in t-PA metabolism. We used human buffy coats to isolate monocytes, cultured them to become macrophages, and studied their interaction with ^{125}I-labeled t-PA. Specific inhibitors and regulators were used to identify the macrophage receptors interacting with t-PA.

MATERIALS AND METHODS.

Materials

Human AB$^+$ serum, and 1-day-old human buffy coats or thrombocyte-poor pooled buffy coats from healthy donor blood, were obtained from the Red Cross Blood Bank (The Hague and Leiden, The Netherlands). Ficoll-Paque (Pharmacia LKB, Biotechnology AB, Uppsala, Sweden) or Lymphoprep (Nycomed Pharma AS, Oslo, Norway) having a density of 1.077 g/mL was used for density gradient centrifugation. Heparin (Leo Pharmaceutical Products, Ballerup, Denmark), bovine serum albumin (BSA; Boseral, Organon Teknika, Boxtel, The Netherlands) or BSA fraction V (Sigma Chemical Co, St. Louis, MO), cell culture medium M199 (Flow Laboratories, Irvine, UK), penicillin/streptomycin (Pen/strep; Boehringer Mannheim, Mannheim, Germany), and sterile buffers were used to isolate and culture the cells. They were cultured in plastic culture plates (Costar Co, Cambridge, MA). Fluorescence-labeled monoclonal antibodies against the human monocyte/macrophage CD14 antigen (CD14-FITC) were obtained from Becton Dickinson Immunocytometry Systems (San Jose, CA). Dexamethasone and lipopolysaccharide extracted from Escherichia coli 0111:B4 were purchased from Sigma Chemical Co. Recombinant melanoma cell-derived t-PA (85% single chain) was prepared at our laboratory, [15,16] and recombinant t-PA (Actilyse) was obtained from Boehringer Ingelheim (Ingelheim, Germany). PPACK (D-Phe-Pro-Arg-chloromethylketone) was from Calbiochem (La Jolla, CA). S-2288 (D-Ile-Pro-Arg-pNA) was purchased from Chromogenix, Mölndal, Sweden. 6-Aminohexanoic acid (6-AHA; Merck Schuchardt, München, Germany), mannose-BSA (mannose 26 mol/mol BSA) and mannan extracted from *Saccharomyces cerevisiae*, prepared by the cetavlon method (Sigma Chemical Co), ovalbumin (Serva, Heidelberg, Germany), mouse monoclonal antibodies against the human placenta mannose receptor (prepared by Barrett-Bergshoeff et al. at our laboratory), and GST-RAP (prepared at our laboratory by Dr J.H. Verheijen using the *Salmonella japonicum* glutathione-S-transferase (GST)-RAP expression plasmid, [17] provided by Dr J. Herz, University of Texas, Southwestern Medical Center, Dallas,TX) were used for inhibition studies.

Cell isolation and cultivation

Buffy coat (100 mL) was diluted with 180 mL phosphate buffered saline (4.5 mmol/L KCL, 228 mmol/L NaCl, 13.5 mmol/L Na_2HPO_4, 2.5 mmol/L KH_2PO_4, pH 7.4) containing 10 U/mL heparin. Portions of 35-mL suspension were underlayered with 14 mL Ficoll-Paque or Lymphoprep. After 30 minutes' centrifugation at 800*g*, the interface was collected and washed (250*g*, 10 minutes) twice with phosphate-buffered saline (PBS: 2.7 mmol/L KCl, 137 mmol/L NaCl, 8.1 mmol/L Na_2HPO_4, 1.46 mmol/L KH_2PO_4), pH 7.4, containing 0.9 mmol/L $CaCl_2$, 0.5 mmol/L $MgCl_2$ and 0.1% BSA. Routinely 1 to 2 x 10^9 cells were obtained. Cells were suspended in M199, and 5x10^6 cells per well were seeded in 12-well plates. Cells were cultured at 37°C in 5% CO_2/ 95% air. After 1 hour, nonadherent cells were removed when the medium was replaced by culture medium (M199 containing 1% glutamine, 50 U/mL penicillin, 50 ug/mL streptomycin, 10% human AB$^+$ serum). Every 2 or 3 days, the medium was refreshed.

For some experiments the cell suspension obtained after gradient centrifugation was further purified using countercurrent flow centrifugation (elutriation) essentially as described by Weiner and Shah. [18] The elutriator (J2-21 centrifuge, JE-6 rotor, standard elutriation chamber; Beckman Instruments Inc, Palo Alto, CA) was first washed with 70% ethanol, then with 0.9% NaCl, and finally with cold (4°C) PBS containing 0.1% BSA (elutriation buffer, EB). Cells washed twice with EB were injected into the elutriation chamber at 2,500 rpm, 10°C, flow rate 2 mL/min. The flow was increased (1 mL/min per 15 seconds) to 14 mL/min. Erythrocytes, thrombocytes, and lymphocytes were collected in 400 mL eluate. The flow was increased again, and monocytes were collected in 100 mL at 17 mL/min, 100 mL at 19 mL/min and 100 mL at 21 mL/min. The fractions were combined, and cells were washed with PBS and resuspended in M199. Routinely, 1 to 2 x 10^8 cells were obtained. Cells were plated at a concentration of 1 x 10^6 cells per well in 24-well plates, and after 1 hour the medium was replaced by culture medium. Every 2 or 3 days, the medium was refreshed.

To analyze the purity of the cell suspensions obtained, a sample of 1 x 10^6 cells was washed with EB and incubated with CD14-FITC (1:20) for 30 minutes at 4°C. Cells were washed once and resuspended in EB, and forward scatter (cell size), side scatter (cell density), and FITC fluorescence were measured using a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems). Data were analysed using FACScan software. The cut-off point for positive (CD14$^+$)/negative cells was determined with reference to the fluorescence of cells not incubated with CD14-FITC.

The extra elutriation step eliminated platelet contamination of the monocytes in culture and did not affect morphological changes during culture. In the experiments described below, no differences were observed between cultured density-gradient-centrifugation-purified or elutriation-purified cells.

Labeling of t-PA

Melanoma and recombinant t-PA were labeled with ^{125}I using the iodogen method.[19] Polyethylene vessels of 1.5 mL were coated with 10 µg iodogen. Next, 10 µg t-PA in 50 mmol/L Tris-HCl, 0.25 mol/L 6-AHA, 1 mol/L NaCl, pH 8, was incubated with 0.5 mCi $Na^{125}I$ for 10 min at room temperature in the vessel. The mixture was transferred to another vessel containing an equal volume of 4 mg/mL KI to avoid non-specific binding of ^{125}I to t-PA. Labeled t-PA was separated from free ^{125}I using a 10-mL Sephadex G25 Coarse column (eluant 50 mmol/L Tris-HCl, 0.01% Tween 80, 1 mg/mL BSA, pH 8). Labeling resulted in approximately 600 cpm/fmol, with 80% recovery of t-PA activity. When indicated, ^{125}I-t-PA was incubated with 2 µmol/L PPACK for 2 hours at room temperature and overnight at 4 °C. The radiolabeled PPACK-t-PA was completely inactivated when tested in the colorimetric activity assay using S-2288. In the studies described below, no differences were observed between labeled or unlabeled melanoma or recombinant t-PA.

Binding of ^{125}I-t-PA

To remove non-adherent cells and serum components, cells (12-well plates) were washed once with PBS, 1% BSA, pH 4, to remove possible receptor ligands and twice with PBS, 1% BSA, pH 7.4, 4°C. ^{125}I-t-PA with or without inhibitors in 500 µL M199, 1% BSA, 0.01% Tween 80 was added. After incubation at 4°C, the medium was removed and the cells were washed with PBS, pH 7.4, containing 0.9 mmol/L $CaCl_2$ and 0.5 mmol/L $MgCl_2$ with (twice) and without (twice) 0.1% BSA, and lysed with 1% Triton X-100 for 15 minutes at room temperature. Radioactivity of the lysates was determined. Binding of ^{125}I-t-PA was corrected for plastic binding in empty parallel wells (10% to 20% of total binding).

Association and degradation of ^{125}I-t-PA

Cells were washed (three times with PBS, 1% BSA, pH 7.4, 4°C) and incubated with ^{125}I-t-PA, with or without inhibitors in 500 µL (12-well plates) or 300 µL (24-well plates) M199, 1%BSA, 0.01% Tween 80, at 37°C in 5% CO_2/ 95% air. After incubation cell media were collected, and trichloroacetic acid (TCA) was added (final concentration 10% wt/vol). Non-degraded ^{125}I-t-PA was precipitated by centrifugation (10 minutes at 15,000*g*). To eliminate the possibility that the cells might deiodinate rather than degrade ^{125}I-t-PA, free ^{125}I was extracted. To the 500 µL TCA-soluble supernatant obtained, 5 µL 40% (wt/vol) KI and 25 µL H_2O_2 30% (vol/vol) were added. After 5 minutes, the free iodine was extracted with 800 µL chloroform. The radioactivity of the remaining ^{125}I-tyrosine (and possibly ^{125}I-peptides) in the upper layer (5 minutes at 15,000*g*) representing degraded ^{125}I-t-PA, was determined. Radioactivity associated with the cells was determined as described for binding at 4°C. Association and degradation were corrected for radioactivity determined in empty parallel wells (less than 5%).

Statistics

Inhibition of the binding or degradation of ^{125}I-t-PA by unlabeled t-PA or other compounds will lead to a decreased value for ^{125}I-t-PA binding or degradation expressed as a percentage of control. No inhibition would result in a constant value of 100% (in spite of the fact that the specific radioactivity is diluted when unlabeled t-PA is tested as inhibitor). At nonsaturating concentrations of labeled compound, a concentration range of inhibitor was added, and the binding or degradation was measured. These inhibition curves were analysed using nonlinear regression analysis with the computer program GraphPAD (ISI Software, Philadelphia, PA), yielding total number of binding sites, apparent dissociation constants (kd), half-maximal inhibitory concentration (IC50), and Hill-slopes. "Goodness of fit" was assessed using actual distances. Using the computer program SOLO 4.0 (BMDP Statistical Software, Los Angeles, CA), the statistical significance of differences was determined with the nonparametric Mann-Whitney test. Two-tailed P <.05% is defined as significantly different. Data are presented as mean ± standard error of the mean (SEM).

RESULTS.

Monocytes were isolated from human buffy coats and cultured to study the interaction of monocytes/macrophages with t-PA. Using density gradient centrifugation, a cell suspension was obtained that contained about 20% $CD14^+$ monocytes. We further purified the suspension by elutriation, and obtained a suspension with approximately 85% $CD14^+$ monocytes. Cells able to adhere to plastic within 1 hour were cultured. The round flat monocytes turned within 2 days into cells with a more contracted appearance and extending pseudopods (macrophages). After the sixth day the first multinuclear giant cells (fused macrophages) started to appear. These results indicated that the majority of the monocytes was differentiated into macrophages between 2 and 7 days of culture. These cells were used for this study.

To determine the presence of t-PA binding sites on the cell membrane of macrophages, the interaction with ^{125}I-t-PA was studied at 4°C. ^{125}I-t-PA appeared to be bound by the macrophages. To determine whether ^{125}I-t-PA was bound specifically by the cells, inhibition of ^{125}I-t-PA binding by unlabeled t-PA was studied (Fig 1). Binding of ^{125}I-t-PA to macrophages was partially t-PA-inhibitable (high affinity binding), and partially non-inhibitable by 350 nmol/L t-PA (low affinity binding). Binding of t-PA to lysine residues is blocked by 6-AHA. Binding of t-PA to the cells was partially inhibited by 6-AHA. Maximal inhibition was obtained at concentrations higher than 10 mmol/L 6-AHA (not shown). In the presence of 20 mmol/L 6-AHA, only high affinity binding of t-PA was found (Fig 1). Nonlinear regression analysis of the t-PA inhibition curves resulted in an apparent kd of 1 nmol/L in the absence of, and an apparent kd of 5 nmol/L in the presence of, 6-AHA. This indicated that 6-AHA did not significantly influence the interaction of t-PA with high affinity binding sites. We found 14×10^3 to 18×10^3 high affinity binding sites per cell. The Hill-slopes calculated for both curves were, respectively, -0.6 and -0.8, suggesting that more than one high affinity receptor was involved in the binding of t-PA.

We observed the interaction of macrophages and t-PA at 37°C to study t-PA catabolism by metabolically active cells. During incubation of 5 nmol/L ^{125}I-t-PA with macrophages, association (bound and internalized ^{125}I-t-PA) reached a maximal level within 2 hours. Association was accompanied by release of ^{125}I-tyrosine into the medium (degradation) from 15 minutes onward. After this lag phase the cells degraded approximately 200 molecules per cell per minute for at least 4 hours. ^{125}I-t-PA degradation was inhibited by chloroquine (100 µmol/L) or NH_4Cl (10 mmol/L) for 75% (not shown), indicating that the degradation was dependent on acidification of the lysosomal compartment.

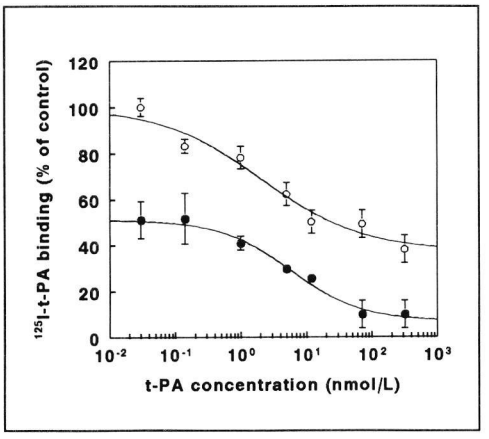


Fig 1 **t-PA binding to macrophages.**
Monocytes/macrophages (4- to 6-day-old) were incubated for 2 hours at 4°C with 1 nmol/L ^{125}I-t-PA with (●) or without (○) 20 mmol/L 6-AHA, and different concentrations of unlabeled t-PA. Data (n=6) were expressed as a percentage of control and were fitted as a sigmoid curve using nonlinear regression analysis.

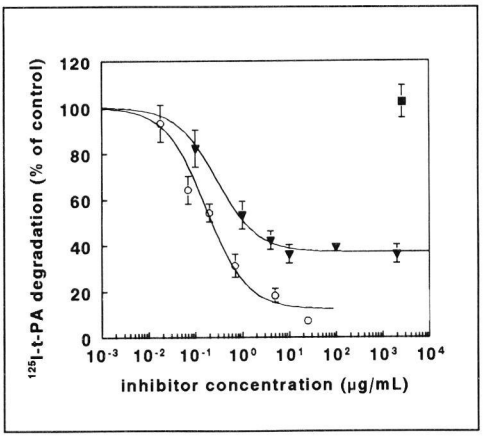


Fig 2 **t-PA degradation by macrophages.**
Monocytes/macrophages (3-day-old) were incubated with 1 nmol/L ^{125}I-t-PA for 3 hours at 37 °C and with different concentrations of unlabeled t-PA (○), different concentrations of mannan (▼), or 2.61 mg/mL (20 mmol/L) 6-AHA (■), and degradation was determined. Data (n=9) were expressed as a percentage of control and were fitted as a sigmoid curve using nonlinear regression analysis.

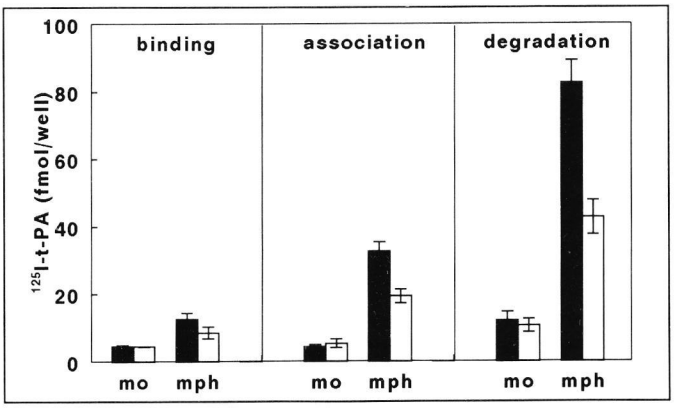
As shown in Fig 2, degradation of ^{125}I-t-PA was fully inhibited by unlabeled t-PA (IC50, 0.2 µg/mL or 3 nmol/L). The IC50 value showed that binding to high affinity binding sites is followed by uptake and degradation. Degradation was not inhibited by 2.61 mg/mL (20 mmol/L) 6-AHA (Fig 2), indicating that low affinity binding of t-PA does not result in uptake and degradation. Mannan, a mannose receptor ligand, partially (maximally 60%) inhibited t-PA degradation (IC50 = 0.3 µg/mL; Fig 2). Other ligands for the mannose receptor, like ovalbumin and mannose-BSA, inhibited degradation to the same extent (not shown).

How is t-PA binding, internalization, and degradation influenced by the differentiation of monocytes to macrophages *in vitro*? Monocytes were cultured for 7 days, and each day, ^{125}I-t-PA binding, association, and degradation in the absence or presence of mannan was determined. One-day-cultured monocytes did bind, internalize, and degrade t-PA (Fig 3). Binding and association gradually increased with time in culture. After a culture period of 2 days (macrophages), the degradation strongly increased, and a maximum was reached at day 3. After this day, the degradation gradually diminished (details not shown). On average (days 2 to 7), t-PA binding, association, and degradation increased, respectively, three, eight, and seven times after differentiation from monocyte to macrophage (Fig 3). Binding, association, and degradation were not mannan-inhibitable at day 1, but they became partially mannan-inhibitable at days 2 to 7. Non-mannan-inhibitable degradation was, on average, four times upregulated after day 1 (Fig 3).

Fig 3

t-PA binding, association, and degradation by human monocytes/ macrophages.

Monocytes were cultured for 7 days. On each day, binding (2 hours, 4°C), association, and degradation (5 hours, 37°C) of 1 nmol/L ^{125}I-t-PA were determined in the absence (■) or presence of 1 mg/mL mannan (□). Data are shown for monocytes cultured for 1 day (mo; n=6), or monocytes cultured for 2 to 7 days (macrophages, mph; n=36).

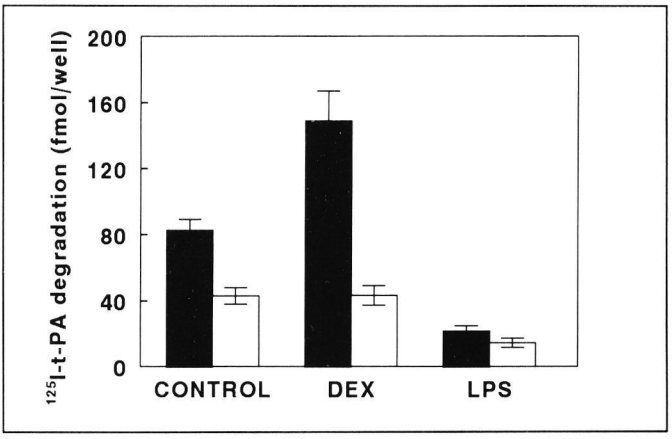


How does suppression or activation of macrophages influence the ability to degrade t-PA? We studied ^{125}I-t-PA degradation by monocytes cultured for 7 days in the presence of the immunosuppressor dexamethasone (DEX) or the activator lipopolysaccharide (LPS). DEX did not have a clear effect on cell morphology. Monocytes cultured with LPS obtained a different morphology from the first day onward. Cells had more and longer pseudopods than nonstimulated cells, while having a smaller centre. Both LPS and DEX had a strong effect on the ^{125}I-t-PA degradation by macrophages (Fig 4): total ^{125}I-t-PA degradation was upregulated two times by DEX and four times downregulated by LPS. Mannan-inhibitable degradation was 2.5 times upregulated by DEX, while non-mannan-inhibitable degradation was not affected. Mannan-inhibitable degradation was five times downregulated by LPS, while non-mannan-inhibitable degradation was, on average, three times downregulated (two times downregulated at days 2 to 3 and five times downregulated at days 4 to 7; details not shown). We concluded that monocyte-derived macrophages express different t-PA receptors which are independently regulated.

Fig 4

Effect of dexamethasone and LPS on macrophage receptor-mediated t-PA degradation.

Monocytes were cultured for 7 days without (control) or with 0.1 µg/mL DEX or 0.1 µg/mL LPS. Average degradation (5 hours, 37°C) of 1 nmol/L ^{125}I-t-PA by 2- to 7-day-old monocytes/macrophages (n=36) in the absence (■) or presence (□) of 1 mg/mL mannan is shown.



Recent studies by Bu et al. [20,21] suggested that t-PA can bind to LRP, which is also expressed on monocytes/macrophages. [22] GST-RAP, a known inhibitor of the interaction of t-PA, t-PA-plasminogen activator inhibitor-1 (t-PA-PAI-1) and other ligands with the LRP, was used to study the possible involvement of LRP. As shown in Fig 5, GST-RAP significantly inhibited degradation of t-PA, and degradation was abolished when 1 mg/mL mannan was coincubated. Half-maximal inhibition of the degradation by GST-RAP was at 5 nmol/L with and 3 nmol/L without mannan.

Similar results were obtained with 3-day-old macrophages cultured without and with DEX or with LPS. GST-RAP (100 nmol/L) inhibited, respectively, 62 ± 2%, 23 ± 6% and 96 ± 2% of t-PA (1 nmol/L) degradation (n=4). Coincubation of GST-RAP (100 nmol/L) with mannan (1 mg/mL) inhibited t-PA degradation for 95 ± 1% in all cases. We concluded that the non-mannan-inhibitable degradation of t-PA by untreated as well as by suppressed or activated macrophages was mediated by the LRP.

Table 1 shows the effect on the t-PA degradation by macrophages of a monoclonal antibody (MoAb) raised against purified human mannose receptor. A control MoAb of the same isotype did not affect t-PA degradation at 10 µg/mL (not shown). Degradation was inhibited by 10 µg/mL anti-mannose receptor MoAb. This did not significantly differ from inhibition with mannan. Coincubation of this MoAb with mannan did not have any additional effect. Coincubation of 10 µg/mL anti-mannose receptor MoAb with 25 nmol/L GST-RAP reduced degradation to 13%. We concluded that the mannan-inhibitable t-PA degradation by macrophages is mediated by the mannose receptor.

No differences were observed in this model between t-PA and PPACK-t-PA (Fig 6), indicating that uptake and degradation of t-PA did not require complex formation with plasminogen activator inhibitors.

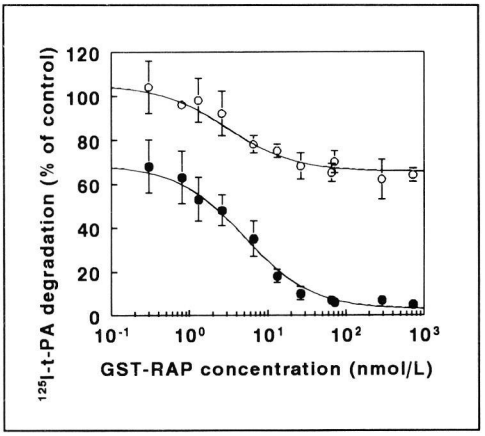


Fig 5 **LRP-mediated t-PA degradation by human macrophages.**

Monocytes/macrophages (3-day-old) were incubated with 1 nmol/L ^{125}I-t-PA for 3 hours at 37°C. Inhibition of t-PA degradation with different concentrations of GST-RAP without (○) or with (●) 1 mg/mL mannan was determined. Inhibition by GST-RAP was statistically significant ($P<.05$) from 6 nmol/L onward. Data (n=4) were expressed as a percentage of control and were fitted as a sigmoid curve using nonlinear regression analysis.

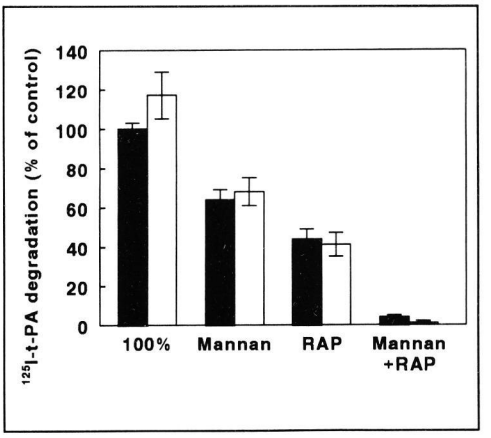


Fig 6 **Degradation of inactivated t-PA by human macrophages.**

Monocytes/macrophages (2- to 6-day-old) were incubated with 1 nmol/L ligand for 4 hours at 37°C. Average degradation of ^{125}I-t-PA (■) or PPACK - ^{125}I-t-PA (□) by the cells without and with 1 mg/mL mannan, 100 nmol/L GST-RAP, or both was determined. Data (n=20) were expressed as a percentage of the ^{125}I-t-PA control.

Tabel 1 **MoAb inhibition of ^{125}I-t-PA (1 nmol/L) degradation by three-day-old macrophages.**

Inhibitor	Concentration	Control	+ Anti-mannose receptor MoAb 10 µg/mL
None		100 ± 3	56 ± 6*
Mannan	1 mg/mL	49 ± 3	45 ± 4
GST-RAP	25 nmol/L	60 ± 4	13 ± 1*

The values represent the residual degradation indicated by the mean percentage of control without inhibitor ± SEM (n=6).
* Significantly different from control.

DISCUSSION

Monocytes differentiated into macrophages within 2 days after plastic attachment. The morphological changes we observed are similar to those observed by others. [23-25] We showed that t-PA bound to high and low affinity binding sites on monocyte-derived macrophages. The high affinity binding sites were ascribed to the mannose receptor and LRP. The low affinity binding sites (kd > 350 nmol/L) were 6-AHA-inhibitable.

Low affinity binding of t-PA has been observed for isolated lipoprotein(a), [26] apo(a) free low-density-lipoprotein, [26] fibrinogen, [26] activated platelets, [27] melanoma cells, [28] monocytes, [29] monocytoid cell lines, [29] endothelial cells, [29] and isolated human liver membrane. [30] Low affinity, 6-AHA-inhibitable binding sites may play an important role for cell bound plasminogen activator activity. [31-33] Our study showed that these binding sites were not essential for the uptake and degradation of t-PA by macrophages, because 6-AHA did not inhibit t-PA degradation.

The apparent kd of t-PA for the high affinity receptors we found on macrophages was 1 to 5 nmol/L (14 x 10^3 to 18 x 10^3 binding sites per cell). These high affinity binding sites mediated t-PA degradation (the half-maximal inhibition of degradation of ^{125}I-t-PA by unlabeled t-PA was 3 nmol/L). This correlates with the fact that the mannose receptor and the LRP are high affinity t-PA receptors. The kd of t-PA binding to the isolated bovine alveolar mannose receptor is 1 nmol/L [34] and to rat liver endothelial cells expressing the mannose receptor 4 nmol/L. [35] With or without PAI-1 involvement, the kd of t-PA binding to LRP ranges from 1-20 nmol/L on various hepatoma cell lines, [20,36-39] smooth muscle cells, [40] and isolated human liver membrane. [30] In our model, no differences were observed between degradation of t-PA and PPACK-inactivated t-PA. As PPACK-inactivated t-PA has a strongly reduced affinity for plasminogen activator inhibitors, [41] the results indicate that complex formation is not necessary for binding to either receptor.

Binding of t-PA at 37 °C (association) was higher than binding at 4 °C, which correlates well with the concept of internalizing ligands and recycling receptors. Indeed, 60-80% of both LRP and mannose receptor are not expressed on the cell surface of macrophages. [42] After ligand binding, both receptors are internalized via coated pits. The receptor dissociates in an acidic environment from the ligand and is recycled to the surface, while the ligand is degraded in a lysosome. There is no competition between the mannose receptor and LRP for the internalization apparatus. [42,43] Lysosomal degradation is chloroquine and NH_4Cl inhibitable, [14,44-46] as observed in our model for t-PA degradation.

LRP is already present on monocytes, and variably expressed on macrophages. [22,47] Monocytes do not express mannose receptors. [48,49] We found that t-PA degradation by 1-day cultured monocytes was not mannan-inhibitable and that this type of degradation was upregulated on macrophages. Mannose receptor mRNA is known to be expressed after 24 hrs in culture. [49] Previously functional mannose receptor activities have not been detected before day 3. [50,51] In our cells, mannose receptor-mediated degradation was observed after 2 days. These differences in expression may be due to culture conditions. [25,52,53]

Macrophage mannose receptor-mediated t-PA degradation was 2.5 times upregulated by DEX. Upregulation has also been found for macrophage mannose receptor expression on the mRNA and on the protein level.[51,54] DEX appeared not to influence LRP-mediated degradation. During LPS stimulation, the mannose receptor was almost not expressed, while LRP was significantly downregulated (five times) after day 3. The downregulation of both receptors by LPS is in line with the observations that LPS downregulates LRP expression after 10 hrs in RAW 264.7 cells [55] and that mouse macrophage mannose receptors are downregulated after activation.[56] The time difference in downregulation that we observed is compatible with the finding that the mannose receptor is more susceptible to downregulation during activation of macrophages than the LRP.[47]

Mannan, ovalbumin, mannose-BSA, all known ligands for the mannose receptor,[14,42,46,57] and a MoAb raised against the isolated human placenta mannose receptor partially inhibited degradation. This proved that degradation was partially mannose receptor-mediated. Degradation of t-PA by macrophages was blocked when mannan and GST-RAP were both present. The 39-kD RAP is a known inhibitor of ligand-LRP interactions.[17,58] LRP has distinct binding sites for different ligands. RAP does not discriminate between the different binding sites on LRP and inhibits binding of all ligands to the receptor with equal efficiency.[17] RAP-inhibitable t-PA degradation by smooth muscle cells is not inhibited by fucose, galactose, mannose, or ovalbumin.[40] We found an IC50 of 3 - 5 nmol/L for the GST-RAP protein on t-PA degradation. This correlates well with the results of Bu et al.,[21,59] who observed an IC50 of 3.3 nmol/L for RAP inhibition of t-PA binding to MH1C1 cells expressing LRP,[21] and a kd of 5 nmol/L for RAP binding to the LRP on HEP G2 cells.[59] We concluded that the mannan-inhibitable t-PA degradation by macrophages is mediated by the mannose receptor and non-mannan-inhibitable degradation is mediated by the LRP.

This is the first report that describes LRP and mannose receptor expressed by human primary cultured cells acting independently on the same ligand. The report shows, in addition, that in macrophages, both pathways are equally efficient in mediating t-PA degradation, without involvement of complex formation of t-PA with inhibitors. Macrophage receptor-mediated degradation may play a significant role in local rather than in systemic clearance of t-PA. When active site blocked t-PA is injected intravenously, only low amounts are found in macrophages (bone marrow, spleen).[57] Nevertheless this human in vitro model seems well suited for evaluating inhibitors of both local and systemic t-PA clearance. Because the major clearance receptors, LRP and mannose receptor, are present, expression can be selectively influenced, and binding can be selectively inhibited.

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CHAPTER 3

Monoclonal antibodies against the human mannose receptor that inhibit the binding of tissue-type plasminogen activator.

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SUMMARY

To study the role of the mannose receptor in cellular uptake and degradation of tissue-type plasminogen activator (t-PA), a set of five monoclonal antibodies (Moab) was generated against the mannose receptor isolated from human placental tissue.

All Moab specifically recognised the 175 kDa mannose receptor in a crude placenta extract, as shown in Western blot analysis. By use of immunohistochemistry, we showed that in human placenta only the Hofbauer cells (fetal macrophages) express the mannose receptor. Epitope competition experiments indicated that the Moab bound to at least two different epitopes on the receptor molecule. Moab 14-3, 14-5, and 15-2, which are directed against one of these epitopes, strongly inhibited the interaction between the purified mannose receptor and t-PA. These Moab also inhibited mannose receptor-mediated degradation of t-PA by cultured human macrophages. The low density lipoprotein receptor-related protein (LRP) mediated t-PA degradation was not affected by the Moab.

It is concluded that the Moab are useful for studying the expression of the human mannose receptor in Western blot and in immunohistochemistry, and for studying the interactions between the human mannose receptor and the mannose-containing ligand t-PA.

INTRODUCTION

The macrophage mannose receptor binds pathogens expressing polymannose structures, and glycoproteins bearing high mannose-type oligosaccharides. After binding the ligand is internalised and degraded in the lysosomes. [1] This receptor is also expressed by liver endothelial cells where it mediates the very rapid endocytosis of its ligands. [2] The receptor contains eight so-called carbohydrate recognition domains. At least three of these domains have mannose-binding properties. [3-5]

Tissue-type plasminogen activator (t-PA) is a serine protease that activates fibrinolysis by converting plasminogen to plasmin which cleaves fibrin to soluble degradation products. [6,7] Because of its fibrin-selective action, t-PA is successfully used for thrombolytic therapy. [8,9] Recombinant t-PA contains one high-mannose- and one or two complex-type oligosaccharides. [10] It is rapidly cleared from the circulation by both the mannose receptor and the low density lipoprotein receptor-related protein (LRP). [11-14] An inhibitor blocking the mannose receptor mediated t-PA degradation could increase t-PA concentrations in blood and thereby stimulate fibrinolysis and thrombolysis.

Ligands for the mannose receptor such as polymannose products (mannan), mannosylated BSA or ovalbumin are good inhibitors of t-PA binding and degradation, [12,15-17] but they may also bind to other mannose binding proteins and may interfere with the function of these binding proteins *in vivo*. [18-19] Monoclonal antibodies might be used to block specifically the mannose receptor, but monoclonal antibodies against this receptor have not yet been described.

We generated monoclonal antibodies (Moab) against the mannose receptor isolated from human placenta. We used these Moab as specific markers for human macrophages using flow cytometry and immunohistochemistry. [20] In the present study we used immunohistochemistry to identify the mannose receptor expressing cells in human placenta from which the receptor was isolated. We studied whether the Moab were able to bind to the mannose receptor and inhibit t-PA binding to the receptor. Furthermore we evaluated whether the Moab were able to inhibit t-PA degradation by human macrophages which are known to express the mannose receptor and the LRP. [15]

MATERIALS AND METHODS

Monoclonal antibodies

Mannose receptor was isolated and purified from human placenta on a mannosylated-albumin-Sepharose column, [21,22] essentially as described earlier. [23,24] Female BALB/c mice were immunised three times, at four-weekly intervals, with 20 µg of purified human mannose receptor. The serum was tested for the presence of antibodies against the mannose receptor in an enzyme linked immunosorbent assay (ELISA) and for the presence of inhibiting antibodies in a t-PA binding assay as described below. All mice immunised had antibodies against the mannose receptor when tested in the ELISA, and all sera contained inhibiting antibodies when tested in the t-PA binding assay.

Cell fusion

Fusion was performed essentially as described earlier. [25,26] Splenocytes (9×10^7) were fused with SP2/0 Ag.14 myeloma cells using 50% polyethylene glycol 4000 (Boehringer Mannheim, Mannheim, Germany). The cell suspension was diluted in culture medium: Dulbecco's modified Eagle medium (Gibco, Paisley, Scotland) supplemented with 10% v/v fetal calf serum containing hypoxantine (100 µM), aminopterin (0.4 µM), thymidine (16 µM) and 10% (v/v) human umbilical vein endothelial cell supernatant (a gift from Dr. P. Koolwijk, produced at our laboratory). The cells were then divided over 96-well microtitre plates (Costar, Cambridge, MA, USA) at a concentration of 0.6×10^5 splenocytes/well and cultured at 37°C in a humidified incubator under 7.5% CO_2. Media of growing cells were screened for antibody production in the ELISA as described below. Cells from positive wells were subcloned by limiting dilution. Positive clones were grown in culture medium and frozen in medium containing 10% (v/v) DMSO at a concentration of 6×10^6 to 10^7 cells/ml.

Production, purification and subclass assessment of the monoclonal antibodies (Moab)

Large quantities of Moab were obtained by *in vivo* (ascites) production in pristane-primed female BALB/c mice. Moab from ascitis in 3.3 M NaCl, 1.5 M glycine, pH 8.9 were bound to a Protein A-Sepharose column (Pharmacia, Uppsala, Sweden), and eluted with 0.1M citrate at pH 4.5. Pooled Moab fractions were dialysed against 20 mM Tris/HCl, pH 7.4, 150 mM NaCl, 5 mM $CaCl_2$. The heavy and light chain subclass of the Moab were assessed by using a Mouse Isotyping kit from Gibco (Paisley, Scotland) according to the manufacturer's instructions.

Western blotting of the mannose receptor

Purified mannose receptor or crude placenta extract [24] was run on 6% SDS polyacrylamide gels under non-reducing conditions. After electrophoresis the gels were blotted overnight at room temperature at 150 mA on nitrocellulose paper in 50 mM borate buffer pH 8.0 containing 10% methanol. Blots were incubated for 2 h with buffer (20 mM Tris/HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.5% Tween 80, 1 mg/ml BSA). The blotting paper was then incubated for 2 h with Moab, and subsequently stained with rabbit-anti-mouse polyclonal IgG peroxidase (Nordic, Tilburg,The Netherlands) and BM blue (Boehringer Mannheim, Mannheim, Germany)

Immunohistochemistry

Placenta tissue was obtained from the Department of Pathology, Slotervaart Hospital, Amsterdam, and snap frozen in liquid nitrogen. As described earlier [20] sections of 8 µm thickness were cut on a Reichert-Jung 2800 frigocut cryostat, transferred to poly-L-lysine coated microscope slides, air-dried and fixed in acetone (10 min room temperature), rinsed in PBS and incubated with 1 µg/ml Moab 15-2 for 30 min at room temperature. After rinsing in PBS (5 min), the sections were incubated with an rabbit-anti-mouse-alkaline phosphatase (Dako A/S, Glostrup, Denmark) for 15 min at room temperature, rinsed with PBS (5 min) and incubated for 30 min at room temperature in the dark with naphtol-AS-MX-phosphate (Sigma Chemical Co., St.Louis MO, USA) and New Fuchsine (Chroma Gezellschaft, Köngen, BRD) as a coupling salt to develop a red reaction product. Finally, the sections were rinsed with aqua dest, counterstained with Mayers hematoxilin and mounted in DePeX mounting medium (BDH, Poole, U.K.). In control sections, no primary antibody was used. The identity of the cells was judged on the basis of their morphology and location in the tissue.

Labelling of Moab and t-PA

Tissue-type plasminogen activator (t-PA), was purified from a recombinant human melanoma cell culture[27] by Dr. J.H. Verheijen.[28] t-PA and Moab at concentrations of respectively 0.1 and 1 mg/ml, were dialysed against 0.1 M $NaHCO_3$, 0.01% v/v Tween 80, pH 8.5 and incubated with NHS-biotin (Zymed Laboratories Inc., South San Francisco, CA, USA) at 30°C (t-PA) or at room temperature (Moab) for 3 h at different molar ratios. Molar ratios of ligand to NHS-biotin were 1 : 200 for t-PA and 1 : 100 for the Moab. After incubation the ligands were dialysed against 20 mM Tris/HCl buffer, pH 7.4 containing 150 mM NaCl, 5 mM $CaCl_2$ and 0.01% (v/v) Tween 80.

For degradation studies, t-PA was labelled with ^{125}I using the iodogen method as described earlier.[15] Labelling resulted in approximately 600 cpm/fmol, with 80% recovery of t-PA activity.

ELISA for mannose receptor antibodies

Polystyrene microtitre plates (Greiner, Frickenhausen, Germany) were coated overnight at 4°C with 100 µl 0.015 µg/ml detergent-free mannose receptor in 20 mM Tris/HCl buffer, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.4. From here onwards the assay was performed at room temperature. The wells were incubated for 0.5 h with "binding buffer" (20mM Tris/HCl, pH 7.4 containing 0.5% (v/v) Tween 80, 150 mM NaCl, 5 mM $CaCl_2$, and 1 mg/ml BSA) and incubated for 1 h with antibodies against the mannose receptor in binding buffer. Bound antibodies were quantified by incubation with peroxidase-conjugated rabbit anti-mouse immunoglobulin (Nordic, Tilburg, The Netherlands) diluted in binding buffer. Between each incubation step the wells were washed 3 times with binding buffer. Bound conjugate was detected by conversion of the chromogenic substrate 3,3',5,5'-tetramethylbenzidine in the presence of H_2O_2.[29] The results were corrected for non-specific binding obtained in wells which were "coated" with buffer without mannose receptor.

Epitope competition assay

To determine whether some of the Moab react with the same or different epitopes on the receptor molecule, we studied the inhibition of labelled Moab binding to the receptor by unlabelled Moab. Plates were coated with mannose receptor as described for ELISA. From here onwards the assay was performed at room temperature. Moab at concentrations of about 1, 10 and 100 times the concentration required for half maximal binding (EC_{50}, see Table 1) in the ELISA, were preincubated for 30 min. Then biotinylated Moab was added at a non-saturating concentration (2 times EC_{50}) and residual binding was measured after 1 h as described for the ligand binding assay (see below).

t-PA binding assay and inhibition by antisera and Moab

Binding of t-PA to immobilised mannose receptor was performed essentially as described earlier.[21] Briefly, mannose receptor (100 µl 0.15 µg/ml) in 20 mM Tris/HCl buffer, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.4 was adsorbed overnight at 4°C to polyvinyl chloride microtitre plates (Flow Laboratories). The wells were incubated for 0.5 h at room temperature with binding buffer. Inhibition of t-PA binding by antisera or Moab was measured by preincubation of the immobilised receptor with varying concentrations of antibodies in binding buffer for 0.5 h. Subsequently a nonsaturating concentration of biotinylated t-PA (100 ng/ml, final concentration) was added and the mixture was incubated at room temperature for 2 h. Bound ligand was quantified by incubation at room temperature for 1 h with alkaline phosphatase-conjugated streptavidine (Amersham, Buckinghamshire, UK). Between each incubation step the wells were washed 3 times with binding buffer. Bound conjugate was detected by measuring the conversion rate of the chromogenic substrate p-nitrophenyl phosphate (Sigma, St. Louis, MO, USA) at 25°C. The results were corrected for non-specific binding obtained in wells which were "coated" with buffer without mannose receptor.

Monocyte isolation and cultivation

Monocytes were isolated from pooled human buffy coats as described earlier.[15] Buffy coat (100 ml) was diluted with 180 ml phosphate buffer (4.5 mM KCl, 228 mM NaCl, 13.5 mM Na_2HPO_4, 2.5 mM KH_2PO_4, pH 7.4) containing 10 units/ml of heparin. Portions of 35 ml suspension were underlayered with 14 ml Lymphoprep™ (Nycomed Pharma AS, Oslo, Norway). After 30 min centrifugation at 800 g, the interface was collected, and washed (250 g, 10 min) twice with phosphate-buffered saline (PBS: 2.7 mM KCl, 137 mM NaCl, 8.1 mM Na_2HPO_4, 1.46 mM KH_2PO_4, pH 7.4) containing 1 mg/ml BSA. Routinely 1 to 2 x 10^9 cells were obtained. This cell suspension was further purified using countercurrent flow centrifugation (elutriation).[15] Elutriation-purified monocytes (approximately 1 to 2 x 10^8 cells, 85% CD14 positive cells) were washed with PBS and re-suspended in culture medium (M199 containing 10 mg/ml glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin, 10% human AB^+ serum). Cells were cultured at a concentration of 2 x 10^6 cells/ml in 24 well culture plates and used on the third day for t-PA degradation studies.

Degradation of ^{125}I-t-PA by macrophages

As described earlier[15] monocytes cultured for 2-7 days on plastic culture plates differentiate and become macrophages. Macrophages were washed three times with PBS (4°C) containing 10 mg/ml BSA, 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$, and incubated for 3 h with 1 nM ^{125}I-t-PA, with or without inhibitors in 300 µl M199, 10 mg/ml BSA, 0.01% Tween 80, at 37°C in 5% CO_2/ 95% air. Mannan extracted from *Saccharomyces cerevisiae*, prepared by the cetavlon method (Sigma St. Louis MO, USA) was used to inhibit

mannose receptor-mediated degradation. In some experiments, receptor associated protein (RAP) was used to inhibit LRP mediated t-PA degradation [15] (prepared at our laboratory by Dr. J.H. Verheijen, using a *Salmonella japonicum* glutathione-S-transferase (GST)-RAP expression plasmid which was kindly provided by Dr. J. Herz, University of Texas, Southwestern Medical Center, Dallas, USA). After incubation, cell media were collected and trichloroacetic acid (TCA) was added (final concentration 10% w/v). Non-degraded ^{125}I-t-PA was precipitated by centrifugation (10 min 15,000 g). To eliminate the possibility that the cells might deiodinate rather than degrade ^{125}I-t-PA, free ^{125}I was extracted. To 500 µl TCA soluble supernatant obtained, 5 µl 40% (w/v) KI and 25 µl H_2O_2 30% (v/v) were added. After 5 min, the free iodine was extracted with 800 µl chloroform. The radioactivity of the remaining ^{125}I-tyrosine (and possibly ^{125}I-peptides) in the upper layer after centrifugation (5 min 15,000 g) representing degraded ^{125}I-t-PA was determined. Degradation was corrected for radioactivity determined in parallel wells without macrophages (<5%).

Statistics

Binding of ligand to the mannose receptor was measured at increasing concentrations of ligand, and plotted against the log of the ligand concentration (binding curve). For inhibition curves, a concentration range of inhibitor with one nonsaturating concentration of labelled ligand was added to the receptor, and the residual binding was measured and plotted against the log of the inhibitor concentration. The sigmoidal binding and inhibition curves were analysed using nonlinear regression analysis with the computer program GraphPAD (ISI Software, Philadelphia, PA, USA), yielding half maximal binding concentration (log EC_{50}) or half maximal inhibitory concentration (log IC_{50}) and their standard error. When at high inhibitor concentration only partial inhibition was observed, the IC_{50} was calculated as the concentration inhibitor where half of the maximal inhibition was observed. "Goodness of fit" was assessed by evaluating the actual distance of the measurements from the fitted line (no weighing).

RESULTS

Five stable cell lines were found and characterised after subcloning, and will be referred to as: 14-2, 14-3, 14-5, 15-2 and 14-14. All five were isotyped as Moab of the IgG_1 subclass with kappa-light chains.

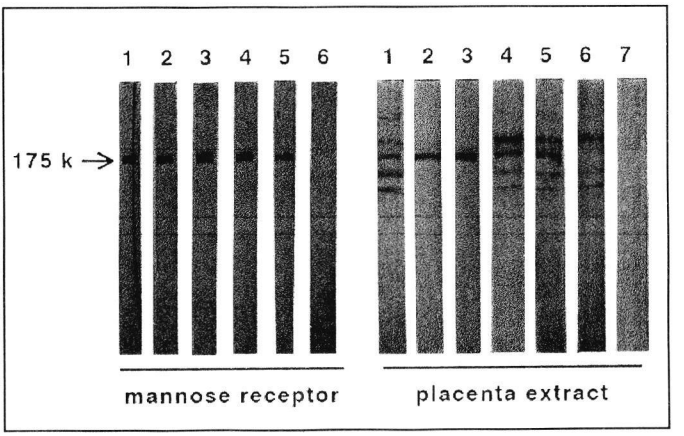
Western blotting

All Moab recognised the purified 175 kDa mannose receptor after SDS-PAGE and transfer to nitrocellulose (Fig 1). In order to study the specificity of the Moab, binding of the Moab to a crude placenta extract from which the receptor was originally purified was analysed. Fig 1 shows that all Moab stained the 175 kDa mannose receptor present in the extract. Moab 14-5 and 15-2 did not stain other proteins. Moab 14-3, 15-14 and 14-2, which were used at higher concentrations than 14-5 and 15-2, showed three additional bands. These bands were also visible with a control Moab not prepared against the mannose receptor at a similar concentration, indicating that they could probably be ascribed to low-affinity- specific or non-specific IgG binding.

Fig 1

SDS PAGE and Western blotting of purified mannose receptor (± 200 ng protein) and crude placenta extract (± 160 µg protein) with the various Moab.

The following Moab were used at concentrations that were sufficiently high to saturate the mannose receptor (see Fig 3). Lanes 1: Moab 14-3 (10 µg/ml); lanes 2: Moab 14-5 (1 µg/ml); lanes 3: Moab 15-2 (0.1 µg/ml); lanes 4: Moab 15-14 (300 µg/ml); lanes 5: Moab 14-2 (50 µg/ml); lanes 6: negative control Moab (150 µg/ml); lane 7: buffer. The position of the 175 kDa mannose receptor is indicated.

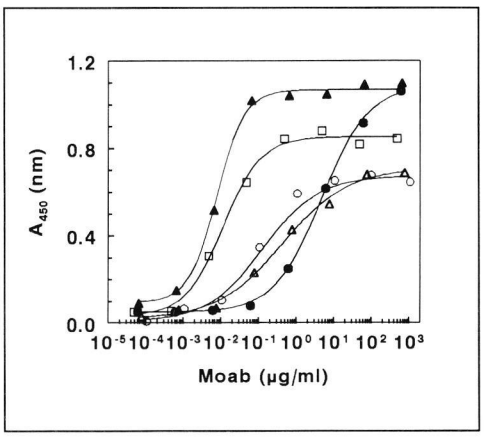


Immunohistochemistry

The Moab presented in this study were also successfully used as specific markers for human macrophages in flow cytometry and in immunohistochemistry.[20] In the present study we used Moab 15-2 to visualize the cells expressing the mannose receptor in human placenta. As shown in Fig 2 (see appendix, page 137), only the Hofbauer cells (fetal macrophages) stained positive for the mannose receptor in human placenta.

ELISA and epitope competition.

The extent of binding of each Moab to the isolated mannose receptor was concentration dependent with EC_{50} values ranging between 0.008 and 4.9 µg/ml (Fig 3, Table 1).



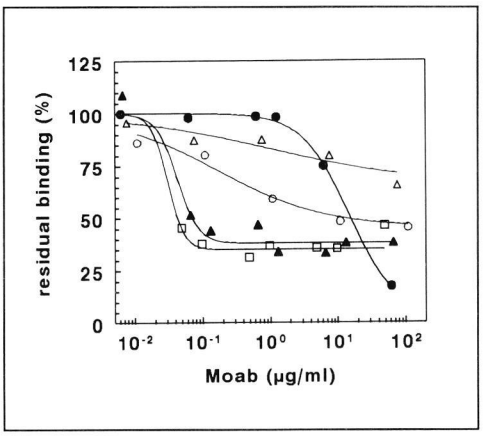


Fig 3 ELISA with monoclonal antibodies.
Dose-response curves of Moab binding to the immobilised isolated mannose receptor, detected with peroxidase-conjugated rabbit anti-mouse IgG. Mean of duplicate measurements are shown. Moab 14-3 (○), Moab 14-5 (□), Moab 15-2 (▲), Moab 15-14 (●), and Moab 14-2 (Δ).

Fig 4 Inhibition of t-PA binding to the mannose receptor.
Effect of Moab on the binding of 0.1 µg/ml biotinylated t-PA to the mannose receptor. Immobilised receptor was preincubated with the Moab, ligand was added, residual binding of the ligand was measured and the inhibition percentage was calculated. Mean of duplicate measurements are shown. Moab 14-3 (○), Moab 14-5 (□), Moab 15-2 (▲), Moab 15-14 (●), Moab 14-2 (Δ).

Results of the epitope competition assay showed that the five Moab bound to at least two different epitopes on the mannose receptor molecule (Table 2). Three Moab (14-3, 14-5, 15-2) apparently recognised the same epitope (epitope cluster I), since they mutually competed for binding to the mannose receptor. A different epitope (epitope cluster II) was recognised by Moab 15-14 and 14-2, they inhibited each other's binding and their binding was not inhibited by 14-3, 14-5 or 15-2. Moab 15-14 however did inhibit subsequent binding of cluster I Moab. This effect cannot be explained by competitive inhibition (which is by definition a mutual effect). Moab 15-14 could have bound to both epitopes and thereby have inhibited the binding of all other Moab but in that case both epitope clusters Moab would have inhibited Moab 15-14 binding partially and a higher signal would have been observed for Moab 15-14 in the ELISA (Fig 3). More probably Moab 15-14 had an effect on the conformation of the receptor resulting in noncompetitive inhibition.[30]

Inhibition of t-PA binding.

In Fig 4 Moab inhibition of t-PA binding to the mannose receptor is shown. Even at high Moab concentrations (1-100 µg/ml), which according to the model of competitive inhibition [30] should have inhibited binding for approximately 95%, only partial inhibition was observed. Cluster I Moab inhibited t-PA binding by about 60%. Cluster II Moab 14-2 showed only slight inhibition (maximallly 30%) whereas Moab 15-14 strongly inhibited t-PA binding at high concentrations. Table 1 summarizes the IC_{50} values for the various Moab and shows that these values agree well with the EC_{50} obtained in the ELISA (Fig 3). In both assays Moab 15-2 and 14-5 showed the highest affinity and Moab 15-14 the lowest affinity for the mannose receptor.

Table 1 **Mannose receptor affinities of Moab functioning as ligand or inhibitor.**

The affinities of the Moab towards the mannose receptor were measured when the Moab were used as ligand in the ELISA (EC_{50}, see Fig 3) and as inhibitor in the t-PA binding assay (IC_{50}, see Fig 4).

ligand/inhibitor	ELISA		t-PA binding assay	
	EC_{50} µg/ml	log EC_{50} ± standard error	IC_{50} µg/ml	log IC_{50} ± standard error
14-3	0.13	-0.88 ± 0.16	0.18	-0.74 ± 0.15
14-5	0.012	-1.91 ± 0.10	0.031	-1.51 ± 0.28
15-2	0.008	-2.09 ± 0.04	0.043	-1.37 ± 0.24
15-14	4.9	0.69 ± 0.05	15	1.17 ± 0.03
14-2	0.44	-0.35 ± 0.15	1.1	0.02 ± 0.37

Table 2 **Moab competition for epitopes on the mannose receptor.**

Immobilised mannose receptor was incubated with a nonsaturating concentration of labelled Moab and a concentration range of nonlabelled Moab. Residual binding of the biotinylated Moab (indicated by *) was measured. Inhibition at a concentration of 100 times EC_{50} (see table 1) of the inhibiting Moab is shown.

Moab	14-3*	14-5*	15-2*	15-14*	14-2*
14-3	+	+	+	=	=
14-5	+	+	+	=	=
15-2	+	+	+	=	=
15-14	+	+	+	+	+
14-2	=	=	=	+	+

+ represents inhibition > 40% ; = represents inhibition < 15%.

Inhibition of t-PA degradation by macrophages.

Cultured macrophages internalise and degrade t-PA after binding to the mannose receptor or LRP. LRP mediated degradation is inhibitable with the receptor-associated protein (GST-RAP), while mannose receptOor mediated t-PA degradation is inhibitable with mannan. [15] This model was used to test whether the Moab were able to inhibit mannose receptor mediated t-PA degradation. As shown in Fig 5A, mannan inhibited total t-PA degradation by 51% and 10 µg/ml Moab 14-5 by 44%. The combination of mannan and Moab 14-5 did not show any additional inhibition of total t-PA degradation, indicating that the LRP mediated degradation was not affected by Moab 14-5. GST-RAP (25 nM) inhibited total t-PA degradation by 40%. t-PA degradation was inhibited with a combination of GST-RAP and mannan by 91% and with a combination of GST-RAP and 10 µg/ml Moab 14-5 by 87 %. Fig 5B shows that inhibition by Moab 14-5 was concentration-dependent and reached at 10 µg/ml about 90% of mannan inhibition in both the presence and absence of GST-RAP. At concentrations of 1 ng/ml to 10 µg/ml Moab 15-14 did not show an inhibitory effect. Moab 14-3 and 14-2 caused an intermediate inhibitory effect (respectively 39 ± 7% and 29 ± 16% at 10 µg/ml), while 15-2 strongly inhibited mannan inhibitable t-PA degradation (68 ± 4% at 10 µg/ml)(not shown).

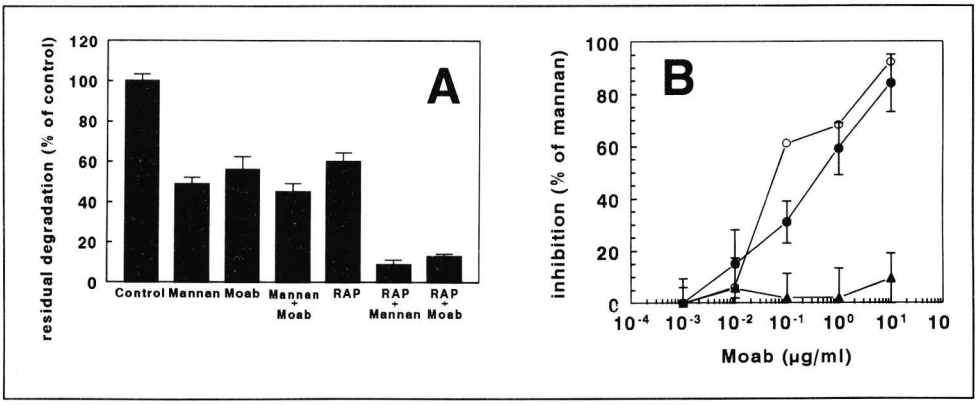


Fig 5 Inhibition of macrophage t-PA degradation by Moab 14-5.

Effect of the Moab on ^{125}I-labelled t-PA degradation by human monocyte derived macrophages. Residual degradation (% of control, n=6, mean ± SEM) was measured in the absence or presence of 1 mg/ml mannan, 10 µg/ml Moab 14-5, 25 nM GST-RAP or their combinations (A). The concentration-dependent effect of the Moab expressed as a percentage of 1 mg/ml mannan inhibition is shown in B. Mannose receptor mediated t-PA degradation in the presence of Moab 14-5 (●), n=6, mean ± SEM, in the presence of Moab 14-5 combined with 25 nM GST-RAP (○), n=2, mean, or in the presence of Moab 15-14 (▲), n=6, mean ± SEM.

DISCUSSION

Five stable cell lines producing Moab with different affinities for the human mannose receptor were obtained. These Moab all reacted in a crude placenta extract with the 175 kDa mannose receptor as shown by the use of Western blot. In another study, we used the Moab to reveal the presence of the mannose receptor on cultured human macrophages using flow cytometry, and showed that with this method we were able to measure different levels of mannose receptor expression. [20] Furthermore we used Moab 14-5 and 15-2 to detect the mannose receptor expressing cells in cryostat sections of human biopsy samples. In thymus, lymph nodes, skin and bone marrow smear only macrophages and young dendritic cells were stained mannose receptor positive. [20]

The immunohistochemistry [20] also showed that the Moab were specific for the mannose receptor since they did not bind to cells (lymphocytes, monocytes, granulocytes, Langerhans cells) that probably contain other mannose binding proteins. [31-33] The Moab also did not bind to cells that express receptors with a configuration similar to the mannose receptor (DEC205 receptor, phospholipase A2 receptor). [34-36] Using Moab 15-2 in immunohistochemistry, we show for the first time that the cells expressing the mannose receptor in human placenta are the Hofbauer cells. These fetal cells have been shown to express many surface antigens also found on monocytes, macrophages and dendritic cells. [37] Many functions of the Hofbauer cells have been proposed; among these are antigen presentation and immune and non-immune phagocytosis. [38] The mannose receptor has been shown to play a role in macrophage phagocytosis [39,40] and antigen uptake by young dendritic cells. [41] The presence of the mannose receptor on Hofbauer cells suggests a similar role of the mannose receptor in the above-mentioned functions of the fetal cells.

On the basis of competition experiments the epitopes of the Moab were analysed in relation to each other and in relation to the binding site of the mannose-containing ligand t-PA. Two epitope clusters were recognised by our set of Moab. Epitope cluster I was recognised by Moab 14-3, 14-5, and 15-2, while epitope cluster II was recognised by Moab 15-14 and 14-2. The affinities of the five Moab differed considerably, but the affinity of each Moab for the receptor was similar in the different assays (Table 1). The Moab with the highest affinities were 15-2 and 14-5, while Moab 15-14 had the lowest affinity.

The mannose receptor contains eight carbohydrate recognition domains (CRD). [3] At least three of them (number 4,5 and 7) are required for high affinity binding of multivalent glycoconjugates. [4] The binding site is however not yet fully delineated. [5] t-PA contains both one high mannose-type and one or two complex-type oligosaccharide chains. [10] Our previous studies with synthetic cluster mannosides showed that t-PA binding to the isolated mannose receptor is completely inhibited by the mannosides. The more mannose residues the inhibitor contains the higher the affinity of inhibition. [42] It is likely that the high mannose-type oligosaccharide of t-PA which has three terminal mannose residues binds to more than one CRD. In this report we showed that t-PA binds to the human mannose receptor and its binding was differently and partially inhibited by the Moab (Fig 4). If the binding sites had been completely identical for the Moab and t-PA the expected inhibition would have been (using the model of competitive inhibition [30]) 97% for Moab 14-3, 100% for Moab 14-5, 100% for Moab 15-2 and 92% for Moab 14-2 at a concentration of 10 µg/ml. Epitope cluster I Moab (14-3, 14-5, and 15-2) inhibited the binding of t-PA to the mannose receptor for 60%, indicating that the epitope and the t-PA binding site partially coincide. Epitope cluster II appears to be more distant from the t-PA binding site, since Moab 14-2 inhibited t-PA binding for about 30%.

Mannose receptor mediated degradation of t-PA by cultured macrophages was inhibited by the Moab in a similar way as t-PA binding to the isolated immobilised mannose receptor. This means that the Moab recognised the receptor not only after isolation and purification, but also in its natural environment on the cell membrane. After incubation of macrophages with labelled Moab we previously obtained a higher signal in flow cytometry at 37°C than at 4°C. This suggests that the Moab are internalised upon binding to the mannose receptor at 37°C, and that free receptor is recycled to the surface.[20] An explanation for the stronger inhibitory effect of Moab 14-5 on mannose receptor mediated t-PA degradation (Fig 5) as compared to mannose receptor binding of t-PA (Fig 4) would be that on macrophages the Moab affect the binding of t-PA to the receptor and also interferes with the process of endocytosis and/or degradation at 37°C.

In this study, we showed that in the presence of 25 mM GST-RAP the 10 µg/ml Moab 14-5 was able to inhibit 87% of total t-PA degradation, and in the absence of GST-RAP 44% of total t-PA degradation. The LRP-mediated t-PA degradation was not affected by Moab 14-5 and the inhibition of the mannose receptor mediated degradation was concentration-dependent. As the inhibition of t-PA degradation by macrophage is representative of the inhibition of *in vivo* clearance,[43] we expect that low concentrations of Moab would be able to inhibit t-PA clearance substantially.

In conclusion, this set of monoclonal antibodies may be useful for the elucidation of ligand-mannose receptor interactions in general and for the specific detection of human mannose receptor in tissue extracts (Western blots) or on cell membranes (immunohistochemistry). In particular the antibodies can be used to block mannose receptor mediated uptake and degradation of t-PA by cells and Fab fragments may be useful for inhibiting clearance of t-PA from the circulation and may thereby stimulate the fibrinolytic system.[44]

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CHAPTER 4

Monoclonal antibodies against the human mannose receptor as a specific marker in flow cytometry and immunohistochemistry for macrophages.

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SUMMARY

Recently we developed mouse monoclonal antibodies (mAb) against the isolated human 175-kDa mannose receptor. In the present study we tested whether these mAb are suitable for the detection of the mannose receptor on cultured macrophages using flow cytometry and on cells in human tissues using immunohistochemistry.

Human monocytes did not react with the mAb in flow cytometry. Mannose receptor expression became detectable on monocytes cultured for 3 days (macrophages), and was maximal from 4 days onward. The mannose receptor was up-regulated on dexamethasone-treated (immunosuppressed) macrophages, and down-regulated on lipopolysaccharide-treated (activated) macrophages.

Immunohistochemically the staining pattern of our mAb was compared with the marker of monocytes/macrophages KP1. In a bone marrow smear, only macrophages were stained with our mAb, whereas all myeloid cells were stained with KP1. In the thymus and lymph node, mannose receptor-positive branched cells (macrophages and dendritic cells) were detected in connective tissue, thymus cortex (not medulla), and in the T cell area (not the B cell area) of lymph nodes, whereas KP1 stained branched cells in all areas. It was concluded that the mAb are useful tools in flow cytometry and immunohistochemistry for the specific detection of cells expressing mannose receptor.

INTRODUCTION

The mannose receptor is known to be expressed on macrophages [1] and probably on liver sinus endothelial cells [2] and airway smooth muscle cells.[3] It plays a role in the immune system for the complement-independent clearance of pathogenic organisms (such as *Candida albicans* and *Pneumocytis carinii*) that have polymannose structures on their membranes. [4] It is suggested that the receptor mediates uptake of the intracellular parasites *Leishmania donovani*,[5] *Tryponosoma cruzi*[6] and *Mycobacterium tuberculosis* [7] by macrophages. Recently it has been suggested that the mannose receptor is involved in the uptake of antigen by human monocyte-derived immature dendritic cells. [8] Furthermore the receptor mediates uptake and degradation of high mannose-type oligosaccharide containing glycoproteins such as lysosomal enzymes [9] and tissue-type plasminogen activator (t-PA).[10,11] Blocking the mannose receptor or increasing its expression on macrophages results in, respectively, increased [12] and decreased extracellular lysosomal enzyme concentrations *in vitro.*[13]

The expression of the receptor is highly regulated and correlates with the functional state of the monocyte/macrophage. Monocytes do not express mannose receptors and resting macrophages express higher levels than activated macrophages. [11,14,15] Monocytes and granulocytes are derived from the same myeloid progenitors in the bone marrow. Commonly used specific markers for human macrophages are also expressed on monocytes (CD14, CD11c, CD68) or on granulocytes (CD11b, CD11c, CD68, CD16). Specific and quantitative measurement of mannose receptor expression might therefore be a good tool for detecting macrophages and for discriminating between various differentiation and activation stages of the macrophage. Mannose receptor expression is frequently assessed by measuring the uptake or binding of receptor ligands. However, the ligand spectrum of the mannose receptor is similar to the ligand spectrum of some other non-macrophage-specific mannose binding proteins which may be present in other cells such as lymphocytes. [16] Binding of labeled mannose receptor ligands to a cell does not prove that the cell is one of the above-mentioned cells

expressing mannose receptor. It has been shown that monocytes and granulocytes are able to bind mannose-BSA, [17] whereas there is no evidence for the presence of the mannose receptor on these cells. [1]

Recently we developed specific monoclonal antibodies (mAb) against the isolated human mannose receptor, which inhibited t-PA binding to the mannose receptor. [18] To our knowledge no mAb against the mannose receptor have been shown to be effective for the detection of human cells expressing mannose receptor. In this study we tested the possibility of using our mAb as a marker for the human macrophage differentiation and activation state in flow cytometry and as a marker for cells expressing mannose receptor in human tissue using immunohistochemistry. We cultured human monocytes in suspension under varying conditions and evaluated mAb binding to the cells by use of flow cytometry. Furthermore we used the mAb in immunohistochemistry on cryostat sections of human tissue and compared the staining patterns with the currently most widely used human macrophage marker KP1, which recognizes the CD68 antigen. [19,20]

MATERIALS AND METHODS

Materials

Human AB$^+$ serum, and thrombocyte-poor pooled buffy coats from healthy donor blood (from six donors with the same blood type combined), were obtained from the Red Cross Blood Bank (The Hague and Leiden, The Netherlands). Lymphoprep^{TM} (Nycomed Pharma AS, Oslo, Norway) with a density of 1.077 g/mL was used for density gradient centrifugation. Heparin (Leo Pharmaceutical Products, Ballerup, Denmark), bovine serum albumin (BSA) fraction V (Sigma Chemical Co., St.Louis MO), cell culture medium M199 (Flow Laboratories, Irvine, UK), penicillin/streptomycin (Boehringer Mannheim, Mannheim, Germany), and sterile buffers were used to isolate and culture the cells. They were cultured in Teflon beakers (Savillex Corp., Minnetonka, MN) to prevent adhesion of the monocytes. [21] Biotin-N-hydroxysuccinimide ester (NHS-Biotin) was supplied by Zymed Laboratories Inc. (South San Francisco, CA). Fluorescein isothiocyanate (FITC), FITC or Phycoerythrin (PE)-labeled mAb against the human monocyte/macrophage CD14 antigen (anti-CD14-FITC, anti-CD14-PE), FITC-, or PE-labeled control immunoglobuline G_1 (IgG_1), and streptavidin-PE conjugate were obtained from Becton Dickinson Immunocytometry Systems (San Jose, CA). FITC labeled mAb against the human monocyte/macrophage/ granulocyte CD11c antigen (anti-CD11c-FITC), mouse serum, propidium iodide (PI), dexamethasone (DEX) and lipopolysaccharide (LPS) extracted from *Escherichia coli* 0111:B4 were purchased from Sigma. Mouse mAb of the IgG_1 isotype (mAb 15-2, 14-5, 14-3, and 14-2) developed against the human placenta mannose receptor [18] were labeled and used for flow cytometry. For immunohistochemical studies rabbit-anti-mouse-alkaline phosphatase and KP1 (mouse monoclonal anti-CD68 isotype IgG1, from Dako A/S, Glostrup, Denmark) were used on cryostat sections of human tissue.

Western blotting of the mannose receptor.

The 175-kDa mannose receptor was isolated and purified from human placenta on a mannosylated-albumin-Sepharose column, essentially as described earlier. [22] Purified mannose receptor was run on 6% sodium dodecyl sulfate (SDS) polyacrylamide gels under nonreducing conditions. After electrophoresis the gels were blotted overnight at room temperature at 150 mA on nitrocellulose paper in 50 mM borate buffer, pH 8.0, containing 10% methanol. Blots were incubated for 2 h with buffer (20 mM Tris.HCl, pH 7.4, 150 mM NaCl, 5 mM ethylenediaminetetraacetate, 0.5% Tween 80, 1 mg/mL BSA). The blotting paper was then incubated for 2 h with mAb, and subsequently stained with rabbit-anti-mouse polyclonal IgG peroxidase (Nordic, Tilburg, The Netherlands) and BM blue (Boehringer-Mannheim).

Labeling of anti-mannose receptor mAb.

For biotinylation 1.31 mg/mL mAb 15-2 was dialyzed against 0.1 M $NaHCO_3$,pH 8.5, and incubated with 27.2 µg/mL NHS-biotin at room temperature for 3 h and dialyzed at 4°C against 20 mM Tris.HCl, pH 7.4, containing 150 mM NaCl, 5 mM $CaCl_2$,and 0.01% (v/v) Tween 80. A whole set of anti-mannose receptor mAb (mAb 15-2, 14-5, 14-3, and 14-2) was labeled with FITC. Approximately 1.5 mg mAb in 1 ml 0.1 M Na_2CO_3 ,pH 9.0, was incubated with 25 µg FITC for 80 min at room temperature in the dark. The unbound dye was separated from the conjugate by gel filtration on a Sephadex G-25 (medium) column. The efficiency of labeling was monitored by estimation of the ratio of fluorescein to protein by calculating the ratio of absorbance at 495 nm/280 nm. FITC-labeled mAb with an absorbance ratio between 0.3 and 1 were used for flow cytometry.

Monocyte isolation and cultivation.

Monocytes were isolated from pooled human buffy coats as described earlier.[11] Pooled buffy coat (100 ml) was diluted with 180 ml phosphate buffer (4.5 mM KCl, 228 mM NaCl, 13.5 mM Na_2HPO_4, 2.5 mM KH_2PO_4, pH 7.4) containing 10 units/mL of heparin. Portions of 35 ml suspension were underlayered with 14 mL Lymphoprep™. After 30 min centrifugation at 800 *g*, the interface was collected and washed (250 *g*, 10 min) twice with phosphate-buffered saline (PBS: 2.7 mmol/L KCl, 137 mmol/L NaCl, 8.1 mmol/L Na_2HPO_4, 1.46 mmol/L KH_2PO_4, pH 7.4) containing 1 mg/mL BSA. Routinely 1 to 2 x 10^9 cells were obtained. This cell suspension was further purified using countercurrent flow centrifugation (elutriation).[11] Elutriation-purified monocytes (approximately 1 to 2 x 10^8 cells, 85% $CD14^+$ cells) were washed with PBS and resuspended in culture medium (M199 containing 10 mg/mL glutamine, 50 U/mL penicillin, 50 µg/mL streptomycin, 10% (v/v) inactivated human AB $^+$ serum). Cells were cultured in suspension at a concentration of 2x10^6 cells/mL in Teflon beakers. Every 3 days the medium was refreshed.

Flow cytometric detection of the macrophage mannose receptor.

The cultured cells were washed with ice-cold binding buffer (50 mM Tris.HCl, pH 7.4, 100 mM NaCl, 2.5 mM $CaCl_2$, 10 mg/mL BSA). After resuspending the cells in binding buffer the cells were incubated with biotinylated mAb and 0.5 mg/mL purified human IgG (purified from human serum by use of protein A-sepharose) for 2 h at 4°C (5 x 10^5 cells/100µL). Cells were washed twice with binding buffer and incubated for 30 min at 4°C with streptavidin-PE. In some experiments the cells were double stained by co-incubating with anti-CD14-FITC or anti-CD11c-FITC. After washing twice in binding buffer, cell scatter and fluorescence were measured with a FACScan (fluorescence-activated cell scanner) flow cytometer, using the program FACScan research software version 2.1 (Becton Dickinson Immunochemistry Systems, San Jose, CA). For studies with FITC-labeled mAb the cells were washed with ice cold PBS containing 1 mg/mL BSA and incubated for 30 min at 4 °C with FITC-labeled mAb in PBS containing 1 mg/mL BSA and 10% (v/v) mouse serum (5 x 10^5 cells/100 µL). The cells were washed twice with PBS containing 1 mg/mL BSA, and resuspended in PBS containing 1 mg/mL BSA and 25 µg/mL propidium iodide at 4°C. Cell scatter and fluorescence were measured using the FACScan flow cytometer. In some studies the same procedure was followed, except that the cells were incubated at 37°C with FITC-labeled mAb.

Analysis of Data

Cell-bound fluorescence was measured using the FACScan flow cytometer, which was calibrated regularly with beads with known scatter and fluorescence intensity. A total of 5000 cells per sample was measured. Labels were excited by a 15 mW Argon laser that produces blue light of 488 nm wavelength. The following five parameters were registered per cell: forward scatter (FSC), reflecting cell size; side scatter (SSC), reflecting the amount of particles inside the cell that scatter the incoming light; green (FITC) fluorescence (FL1); orange (PE) fluorescence (FL2); and red fluorescence (FL3). Spectral overlap between the green, orange and red fluorescence was electronically compensated. PI only penetrates through membranes of dead cells and stains their DNA. Dead cells, cell debris, and lymphocytes had a different FSC and SSC from living monocytes or macrophages. Only cells with an FSC and an SSC of living monocytes/ macrophages (>95% $CD14^+$ cells, < 5% PI^+ cells) were used for the quantitative measurement of the fluoresence caused by bound anti-mannose receptor mAb. Values of fluorescence were obtained as channel numbers and the mean channel number of the (selected) cell population was calculated. For the discrimination of populations of cells the fluorescence was measured in a log mode with each channel number reflecting a log arbitrary fluorescence unit. When measurements were performed in a linear mode, the channel numbers reflected arbitrary fluorescence units. Because log amplifiers are not perfectly logarithmic and the number of channels may differ per decade[23] a calibration curve was made of the mean channel number of cells labeled with different amounts of anti-CD14-FITC or anti-CD14-PE measured in the linear mode and in the logarithmic mode. When measurements were performed in the log mode, this calibration curve was used to calculate the mean arbitrary fluorescence units from the mean channel number .

Immunohistochemistry.

Tissues were obtained from biopsy samples (Department of Pathology, Slotervaart Hospital, Amsterdam) and snap frozen in liquid nitrogen. A modification of the immuno alkaline-phosphate (AP) method described by Li et al.[24] was used. Sections of 8 µm thickness were cut on a Reichert-Jung 2800 frigocut cryostat, transferred to poly-L-lysine coated microscope slides, air dried, and fixed in acetone (10 min at room temperature), rinsed in PBS, and incubated with primary antibody for 30 min at room temperature. After rinsing in PBS (5 min), the sections were incubated with an alkaline-phosphatase-conjugated secondary antibody for 15 min at room temperature, rinsed with PBS (5 min), and incubated for 30 min at room temperature in the dark with naphtol-AS-MX-phosphate (Sigma) and New Fuchsine (Chroma Gezellschaft, Köngen, BRD) as a coupling salt to develop a red reaction product. Finally the sections were rinsed with aqua dest, counterstained with Mayers hematoxilin, and mounted in DePeX mounting medium (BDH, Poole, UK). Serial dilutions of mAb 14-5 and 15-2 in PBS were tested for reactivity and finally used at 1 µg/mL. In control sections no primary antibody was used. The identity of the cells was judged on the basis of their morphology and location in the tissue. The mAb were also tested on paraffin-embedded tissue sections. Only mAb 15-2 showed some reactivity with these sections but the staining was granule-like and not clearly associated with the cells, therefore only cryostat sections were used in this study.

RESULTS

Western blot

To show that our mAb recognized the 175-kDa human mannose receptor we performed a Western blot. As shown in figure 1 the mAb 15-2 and 14-5, and not the control mAb, specifically stained the 175-kDa mannose receptor isolated and purified from human placenta (the antigen we used to immunize mice [18]).

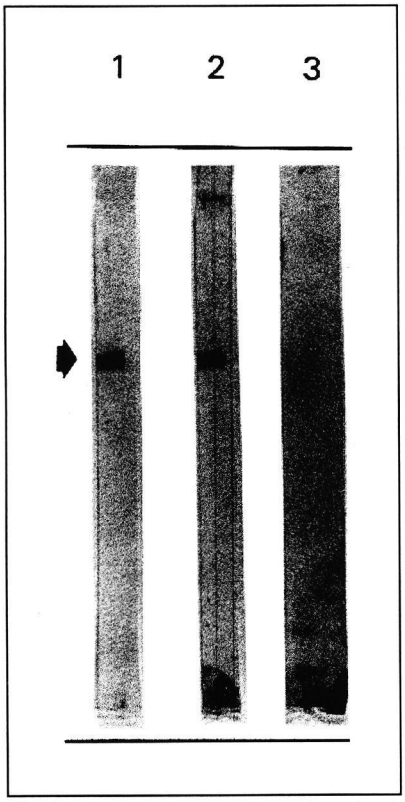

Fig 1 SDS PAGE and Western blotting of purified mannose receptor.
Lane 1, mAb 14-5; lane 2, mAb 15-2; lane 3, isotype-matched control mAb. The arrow indicates the position of the 175 kDa mannose receptor.

Facs analysis of monocyte/macrophage differentiation

Isolated human monocytes (85% $CD14^+$) were cultured in suspension. Cells were stained each day for mannose receptor and CD14 or CD11c using, respectively, biotinylated mAb 15-2 combined with streptavidine-PE and anti-CD14-FITC or anti-CD11c-FITC. In the presence of 10% mouse serum or 0.5 mg/mL IgG no binding of labeled control IgG_1 was detectable throughout the experiments. A typical example of the monocyte, lymphocyte, and macrophage staining with anti-CD11c (fluorescence 1) and mAb 15-2 (fluorescence 2) is shown in Fig 2A. The total population of cells consisted of monocytes/macrophages, lymphocytes, and cell debris. Each population was analysed separately using electronic gating. Monocytes/macrophages (gate 1) were distinguished from lymphocytes and cell debris (gate 2) by their size (forward scatter) and amount of internal particles (side scatter). We show the reactivity of the cultured gate 2 cells because they are representative for lymphocyte staining; fresh lymphocytes isolated separately from the pooled buffy coats appeared in the same gate and reacted in the same way as cultured gate 2 cells to the mAb tested below (not shown). More than 95% of the gate 1 cells were stained with anti-CD14 or anti-CD11c on each day studied. Gate 1 macrophages (cultured 4 days), but not monocytes (cultured 1 day), were stained with mAb 15-2. No binding of mAb 15-2, anti-CD14, or anti-CD11c to lymphocytes was observed during the whole culture period. The fluorescence caused by mAb binding compared with the blank fluorescence (mainly autofluorescence) is shown in the histograms (Fig 2B).

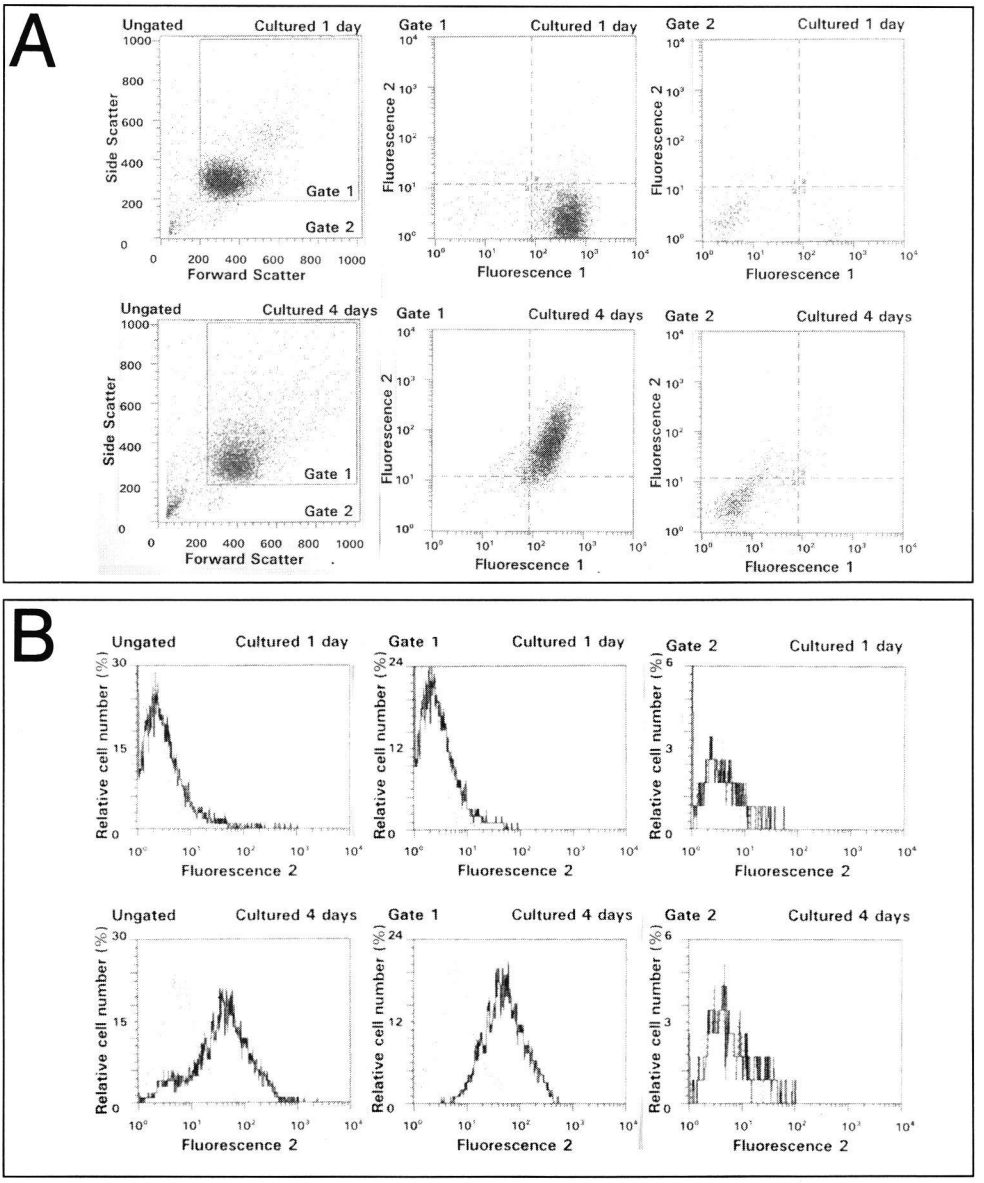

Fig 2 Facs analysis of mAb 15-2 binding to human lymphocytes, monocytes and macrophages.

Monocytes were cultured for 1 or 4 days and incubated at 4°C with or without 10 µg/mL biotinylated mAb 15-2 followed by an incubation with streptavidin-PE (fluorescence 2) and anti-CD11c-FITC (fluorescence 1). Gates were set on populations based on the forward scatter (cell size) and side scatter (internal cell particles). Gate 1 contained monocytes/macrophages, whereas gate 2 contained lymphocytes and cell debris. Total and scatter-gated dot plots are shown, with the dotted cross indicating the limits of the blank fluorescence (A). Overlay histograms (relative cell number versus log fluorescence 2) of cells incubated with mAb 15-2 plus streptavidin-PE and anti CD11c-FITC (line) and control cells incubated with streptavidin-PE and anti-CD11c-FITC (dotted line) of the total and scatter-gated cell populations are shown in B.

The mean fluorescence of gate 1 cells was calculated for each day during culture (Fig 3). The mannose receptor was not detectable on 1- to 2- day cultured monocytes, and became detectable after culturing the monocytes for 3 days. Binding of the mAb was concentration dependent, and expression of the mannose receptor remained stable from day 4 onward. Compared with these results obtained with biotinylated mAb 15-2, similar results were obtained with FITC-labeled mAb 15-2, 14-5, and 14-3, which recognize the same epitope, and with mAb 14-2, which recognizes a different epitope on the mannose receptor. [18] The lymphocytes (gate 2 or isolated from buffy coats by use of elutriation) did not react with any of these mAb (not shown). The specificity of binding of mAb 14-5, 14-3, and 14-2 was demonstrated on 6- to 9-day-old cells. Fig 4 shows that the unlabeled mAb were able to compete for the binding of the corresponding FITC-labeled mAb. No inhibition was observed with 600 µg/mL control IgG_1.

When living macrophages were incubated with FITC-labeled mAb 15-2 at 37°C a higher signal was obtained than at 4°C (Fig 5A). Fig 5B shows that the binding at 37°C and at 4°C of labeled mAb 15-2 was specific because it was inhibited with excess unlabeled mAb 15-2. Because the inhibition with cold ligand occurred at similar concentrations at both temperatures, the increased signal appeared not to be due to increased affinity for the receptor at 37°C. The higher signal at 37°C suggests accumulation of labeled mAb inside the cell, which is compatible with internalisation of the mAb-receptor complex, release of the mAb inside the cell, and recycling of the receptor. [25]

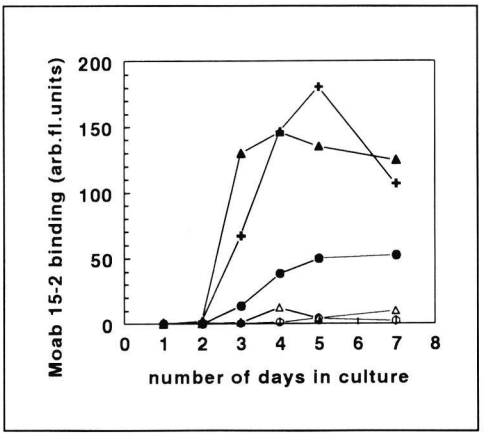

Fig 3 Binding at 4°C of different concentrations of biotinylated mAb 15-2 to cells cultured for 1 - 7 days. Binding was detected by use of streptavidin-PE and fluorescence was measured in a log mode. mAb binding to scatter gate 1 cells was expressed as mean arbitrary fluorescence units (calculated using a calibration curve; see Materials and Methods). Concentration mAb 15-2: 10 µg/mL (▲), 5 µg/mL (✚), 1 µg/mL (●), 0.1 µg/mL (Δ), blank (○).

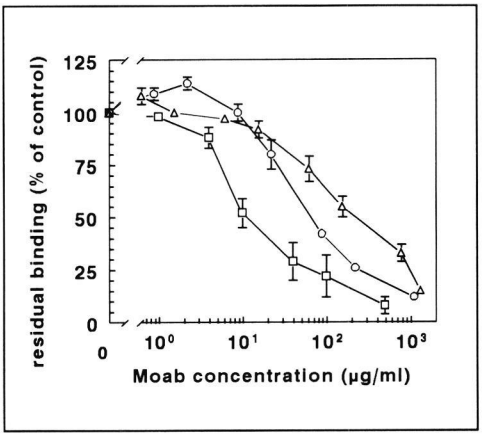

Fig 4 Binding at 4°C of different mAb to monocytes/ macrophages.
Monocytes cultured for 6-9 days were incubated at 4°C with FITC-labeled anti-mannose receptor mAb 14-5, 14-3, or 14-2 (25-50 µg/mL) and different concentrations of the corresponding unlabeled mAb. Residual mAb binding to the scatter gate 1 cells was measured in a linear mode, and expressed as a percentage of control (mean ± standard error of the mean, n=3). Symbols are as follows: mAb 14-5 (□), mAb 14-3 (○), and mAb 14-2 (Δ).

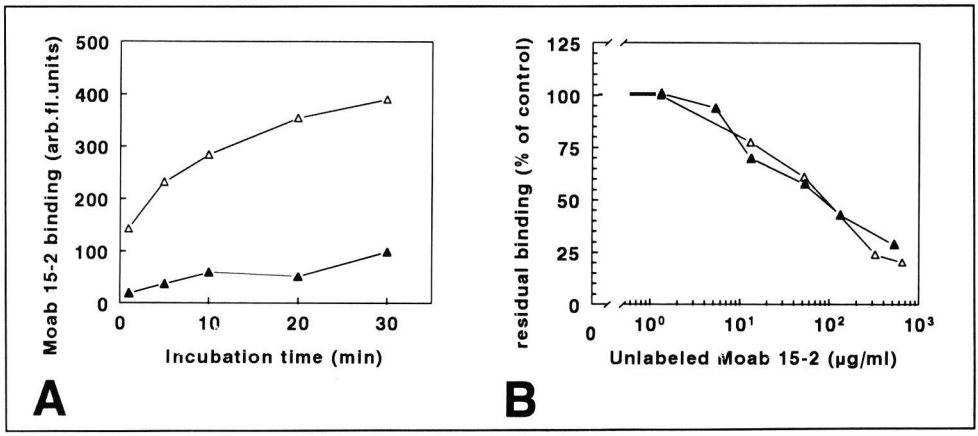

Fig 5 Temperature dependency and specificity of the interaction between mAb 15-2 and monocytes/macrophages cultured for 5 - 7 days.

Cells were incubated with 35 µg/mL FITC-mAb 15-2 and 0.5 mg/mL purified human IgG in PBS containing 1 mg/mL BSA. FITC-mAb 15-2 binding to scatter gate 1 cells was measured in a log mode and expressed as mean arbitrary fluorescence units (calculated by use of a calibration curve, see Materials and Methods). Incubations were performed at 4°C (▲) or at 37°C (Δ) for various time periods (A), and for 30 min at 4°C (▲) or at 37°C (Δ) in the presence of increasing concentrations of unlabeled mAb 15-2 (B)

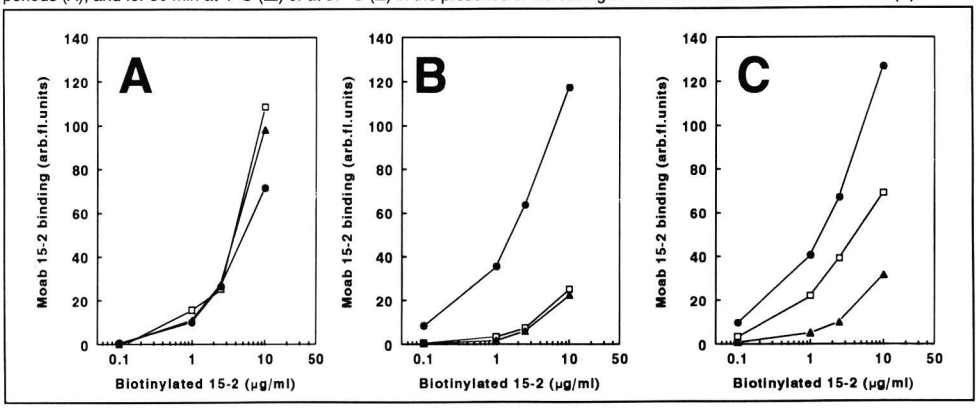

Fig 6 Effect of DEX or LPS on biotinylated mAb 15-2 binding to monocytes cultured for 3 or 4 days.

Binding was detected by use of streptavidin-PE and fluorescence was measured in a log mode. mAb binding to scatter gate 1 cells was expressed as mean arbitrary fluorescence units (calculated with the use of a calibration curve, see Materials and Methods). Cultured monocytes were treated with or without 0.1 µg/mL DEX or LPS on day 3, and measured at day 4 (A). Monocytes were continuously treated from day 0 onward and measured at day 3 (B) and on day 4 (C). Symbols are as follows: non-treated (□), DEX-treated (●), LPS-treated (▲).

Facs analysis of activated/immunosuppressed macrophages

To assess whether it was possible to detect different levels of mannose receptor expression by flow cytometry using the anti-mannose receptor mAb, we treated the monocytes/macrophages with DEX or LPS in order to up- or down-regulate the receptor and measured scatter, autofluorescence, anti-CD11c, anti-CD14, and mAb 15-2 binding. Cells were treated with DEX or LPS on day 3 and after 24 h mAb 15-2 binding was measured (Fig 6A). No differences were observed from non-treated cells.

Then, from day 0, monocytes were treated with these agents and binding was measured at day 3 (Fig 6B) and day 4 (Fig 6C). The cells were differently affected by the two agents. Compared to that of nontreated cells the average autofluorescence, forward, and side scatter of the cells was higher and the CD14 expression lower when stimulated 3 or 4 days with DEX, whereas the side scatter was higher and CD11c expression was lower when stimulated for 4 days with LPS (not shown). mAb 15-2 binding was up-regulated by DEX after 3 and 4 days in culture, and down-regulated (not up-regulated like non-stimulated macrophages) by LPS after 4 days in culture. Throughout the experiments the cells expressing mannose receptor displayed a normal fluorescence distribution and no population subsets were distinguished.

Immunohistochemistry

Table 1 shows a comparison of cell staining with KP1 and cell staining with mAb 15-2. mAb 14-5 was also tested and stained the same cells as mAb 15-2 (not shown). Very different staining patterns of mAb 15-2 and KP1 were observed. In a cytospin of bone marrow cells, the granulocytes, monocytes, and macrophages (all myeloid cells) were stained with KP1, whereas only the macrophages reacted with mAb 15-2. In the thymus KP1 reacted with branched cells (macrophages and dendritic cells) in every area, with rounded cells (monocytes, granulocytes?) and with the endothelium of small blood vessels in the medulla. mAb 15-2 predominantly stained branched cells in the cortex and only a few branched cells in the medulla. Both mAb reacted with the branched cells lying along the connective tissue septa. In the lymph node KP1 reacted with branched cells in every area, whereas mAb 15-2 predominantly reacted with branched cells in the subcapsular sinus, the T cell area, and the medulla. In the skin, KP1 stained branched cells in the dermis and the Langerhans cells in the epidermis, whereas mAb 15-2 only stained the branched cells in the dermis and not the Langerhans cells in the epidermis. Representative areas of thymus and lymph node stained by KP1 or mAb 15-2 are shown in Fig 7 and 8.

Table 1: Comparison of cell staining with KP1 or mAb 15-2 in human tissue.
+ strong staining, - no staining, - (+) very few cells stained.

TISSUE	AREA	CELL TYPE	KP1	15-2
Bone marrow smear		lymphocytes	-	-
		granulocytes	+	-
		monocytes	+	-
		megakaryocytes	-	-
		macrophages	+	+
Thymus	cortex	branched cells[a]	+	+
	connective tissue trabecula	branched cells	+	+
	medulla	rounded cells	+	-
		branched cells	+	- (+)
		endothelium	+	-
Lymph node	subcapsular sinus	branched cells	+	+
	T cell area	branched cells	+	+
	B cell area			
	mantle zone	banched cells	+	- (+)
	germinal centre	branched cells	+	- (+)
	medulla	branched cells	+	+
Skin	epidermis	Langerhans cells	+	-
	dermis	branched cells	+	+

[a]The branched cells observed can be macrophages or dendritic cells (called interdigitating cells in the T cell area or follicular dendritic cells in the B cell area). Both celltypes are known to be present in lymphoid and non-lymphoid tissues, and macrophages are morphologically not different from dendritic cells when viewed by light microscopy (see Discussion).

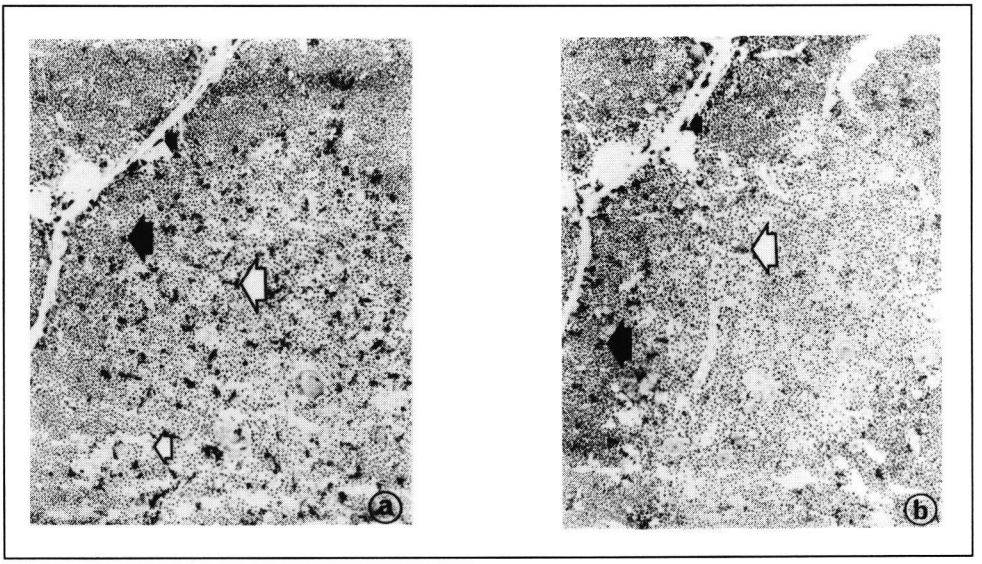

Fig 7 Staining patterns of KP1 (a) and mAb 15-2 (b) in cryostat sections of human thymus. In the middle, the thymus medulla with many KP1 and few mAb 15-2 positive cells is shown (branched cells, large white arrow; endothelium small white arrow). In the periphery the thymus cortex with KP1 and mAb 15-2-positive cells (branched cells, large black arrow) and the connective tissue septa with KP1 and mAb 15-2-positive cells (branched cells, small black arrow) are shown.

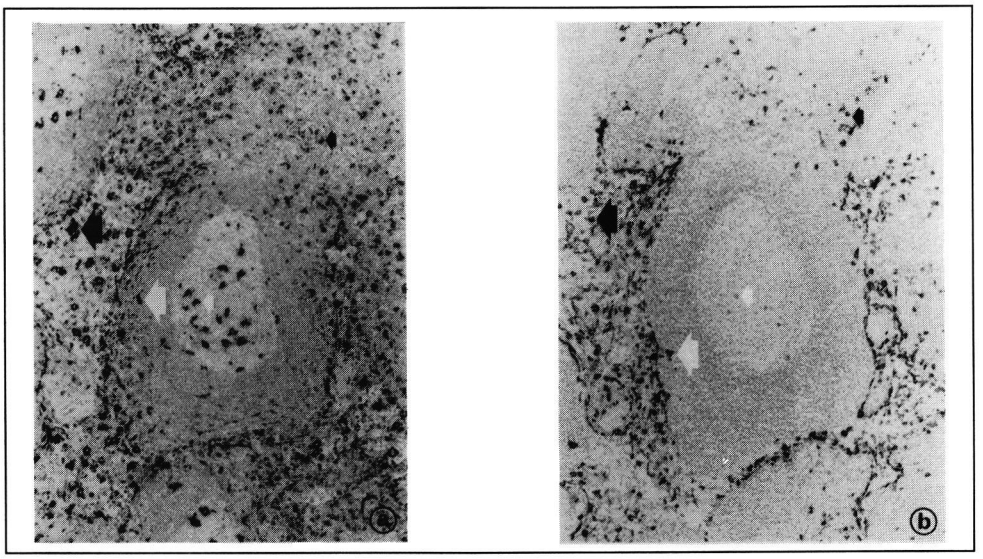

Fig 8 Staining patterns of KP1 (a) and mAb 15-2 (b) in cryostat sections of human lymph node. In the middle the B cell area, follicle with germinal centre and mantle zone, containing many KP1 and few mAb 15-2-positive cells is shown (branched cells, germinal centre, small white arrow; mantle zone, large white arrow). In the periphery the T cell area with KP1 and mAb 15-2 positive cells (branched cells, small black arrow) and the medulla with KP1 and mAb 15-2 positive cells (branched cells, large black arrow) are shown

DISCUSSION

In this study we describe the use of mAbs against the human mannose receptor to specifically detect human cells expressing mannose receptor in flow cytometry and immunohistochemistry

Human monocytes differentiate to macrophages depending on the culture conditions within a few days. [21,26] Mannose receptor-mediated binding and degradation of ligands is observed after culturing the monocytes for a few days. [4,11] Our mAbs, which were able to detect the 175-kDa mannose receptor isolated from human placenta in Western blots, proved to be suitable for specific and quantitative detection of cells expressing mannose receptor using flow cytometry. Using the mAb we showed that the mannose receptor is absent on lymphocytes and monocytes and is up-regulated on monocytes during differentiation to macrophages.

Binding of the mAb was concentration dependent, and was inhibited by the corresponding unlabeled mAb. At 37°C mAb 15-2 possibly was internalized and the free receptor was recycled because, compared with the binding at 4°C, higher levels of fluorescence were observed without any change in the mAb affinity for the receptor. This is consistent with other studies which state that approximately 80% of the macrophage mannose receptor is not expressed on the cell membrane due to the internalization and recycling of the receptor. [25]

Down-regulation by LPS and up-regulation by DEX of the macrophage mannose receptor activity have previously been demonstrated. [11,15,27-29] LPS down-regulates and DEX up-regulates macrophage mannose receptor-mediated t-PA degradation. [11] We showed that it was possible with mAb 15-2 to discriminate between non-stimulated, LPS stimulated and DEX stimulated macrophages. Down-regulation by LPS of both mannose receptor and CD11c was observed on cells cultured for 4 days. LPS irreversibly inactivates the receptor without affecting total receptor biosynthesis by an unknown mechanism. [27] mAb 15-2 recognizes a site close to the t-PA binding site because it is able to inhibit t-PA binding to the mannose receptor. [18] The LPS mediated down-regulation of mAb 15-2 binding observed does not necessarily mean that the whole receptor was down-regulated; it could have been caused by binding site masking or destruction. The correlation of decreased mAb 15-2 binding with decreased receptor function suggests that mAb 15-2 binding in flow cytometry reflects functional mannose receptor expression.

DEX up-regulates the mannose receptor by up-regulation of receptor mRNA followed by receptor synthesis [28] as does interleukin-4.[29] Granulocyte-monocyte colony-stimulating factor and interleukin-4 treatment of monocytes results in differentiation to cells with up-regulated mannose receptors and many characteristics of immature dendritic cells. [8,30] Macropinocytosis and mannose receptors are used by these cells for the efficient capture of antigens. [8] In our experiments mannose receptor up-regulation by DEX was observed on the third day in culture. It may be possible that the higher expression of mannose receptor on our DEX-treated monocytes was an indication of differentiation of the monocytes to cells with characteristics of immature dendritic cells.

Immunohistochemistry showed that our mAbs (mAb 15-2 and 14-5) did not recognize cells that are known to contain other mannose binding proteins with a similar ligand spectrum, or receptors with a similar configuration to the mannose receptor. mAb 15-2 did not stain lymphocytes that have mannose binding protein [16] and no lymphocytes or vascular endothelial cells that express pancreatic group I phospholipase A2 receptor [31] (a receptor analogous to the mannose receptor[32]). The mAb did not stain thymic epithelial cells that may express the DEC-205 receptor (a receptor analogous to the mannose receptor [33]). Although freshly isolated Langerhans cells are able to internalize mannose-BSA, no 175-kDa receptor is found in Western blots. [34] We did not find mannose receptor positive Langerhans cells in the epidermis, indicating that a different mannose binding protein mediates the uptake of mannose-BSA. Similarly monocytes and granulocytes, which are able to bind mannose-BSA [17] and do not contain the 175-kDa mannose receptor, [1] were not stained with mAb 15-2. KP1, although commonly used as a macrophage marker, also stains other myeloid cells (monocytes, granulocytes) besides macrophages and some endothelial and epithelial cells. [20] We showed a different staining pattern for KP1 and mAb 15-2.

It has been suggested that the mannose receptor plays a role in the uptake of antigen followed by antigen presentation by dendritic cells, Langerhans cells, and macrophages *in vitro*. [8,34,35] We showed that most of the known cells of the dendritic cell family (Langerhans cells, follicular dendritic cells in the B cell areas of the lymph node and the interdigitating dendritic cells in the thymus medulla) did not express the mannose receptor, whereas we did find mannose receptor-positive branched cells in the dermis, lymph node T cell areas, and the thymus cortex. Dendritic cells exist in two stages of maturation. Immature cells are present scattered throughout the body. When they pick up and process antigen they subsequently move to the T cell areas of secondary lymphoid organs. Somewhere during this process (location unknown) they mature; they lose their antigen-capturing capacity and become cells that trigger the naive T cells recirculating through the T cell areas. [36]

Monocytes cultured in the presence of granulocyte-monocyte colony-stimulating factor and interleukin-4 become immature dendritic cells that express the 175 kDa mannose receptor while being able to capture antigens, and that down-regulate the mannose receptor when they are stimulated with maturation-inducing agents like tumor necrosis factor α and LPS. [8] Stimulation of these cells with tumor necrosis factor α results in decreased antigen capture capacity and increased stimulatory capacity in a mixed lymphocyte reaction. [30] We suggest that the mannose receptor-positive (and KP1 positive) branched cells seen in the tissues represent resident macrophages and immature dendritic cells, and the mannose receptor-negative (and KP1 positive) branched cells represent activated macrophages and mature dendritic cells. Thus our mAb may be useful in combination with other markers to study the process of dendritic cell maturation in human tissues.

Because the mannose receptor is thought to be involved in the non-opsonic degradation of pathological organisms, [4] and in the internalization of parasites that manage to survive and replicate inside the macrophage, [5,6,7] our mAbs may offer new insights into the role of the mannose receptor in various diseases.

In conclusion the mannose receptor is a specific marker for different types of macrophages. Our mAb proved to be a useful tool for detecting the 175-kDa mannose receptor in Western blots, for discriminating resting macrophages from immunosuppressed and activated macrophages using flow cytometry, and for detecting cells expressing mannose receptor in cryostat sections of human tissues.

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CHAPTER 5

Expression of the human mannose receptor under physiological and pathological conditions. An immunohistochemical study using monoclonal antibody 15-2

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SUMMARY

The 175 kDa mannose receptor has been shown to be involved in different processes such as clearance, antigen uptake and fusion of macrophages into giant cells. The mannose receptor expression on macrophages is downregulated by activation and upregulated by alternative activation of macrophages *in vitro*. Recently we developed a specific monoclonal antibody (mAb) that recognizes the human mannose receptor, mAb 15-2. In the current report the expression of the human mannose receptor under physiological and pathological conditions was immunohistochemically studied in tissues by the use of mAb 15-2.

Under physiological conditions, the mannose receptor was expressed on resident macrophages, liver and splenic sinusoidal endothelial cells, spermatozoa and spermatids. In tuberculosis and leprosy, in rheumatoid arthritis and in breast carcinoma the macrophages were probably activated as the mannose receptor expression by macrophages appeared to be downregulated. In contrast, in a foreign body reaction the macrophages were probably alternatively activated as the mannose receptor expression by macrophages appeared to be upregulated.

It is concluded that only a few human cell types express the mannose receptor. As previously shown *in vitro*, the mannose receptor expression on macrophages probably reflects the type of macrophage activation *in vivo*. mAb 15-2 is a specific tool to study the mannose receptor expression in human tissues.

INTRODUCTION

The 175 kDa mannose receptor is expressed by macrophages *in vitro*. Antagonists of the mannose receptor inhibit the uptake and degradation of high mannose-type oligosaccharide containing glycoproteins such as lysosomal enzymes [1] and tissue-type plasminogen activator (t-PA). [2] They also inhibit the complement-independent uptake of pathogenic organisms that have polymannose structures on their membrane. [3] Mannose receptor-mediated uptake results in either intracellular degradation of organisms such as *Candida albicans*, or intracellular survival of parasites such as *Mycobacterium tuberculosis*. [3] It also plays an important role in the antigen uptake and processing by dendritic cells [4,5] and fusion of macrophages into foreign body-type giant cells after interleukin-4 stimulation. [6] Furthermore the mannose receptor expressed on other cell types appears to mediate the plasma clearance of high mannose-type oligosaccharide containing glycoproteins in the liver, [7,8] the homing of lymphocytes to the spleen, [9,10] sperm fertility [11] and the induction of alveolar smooth muscle cell proliferation. [12,13]

It has been suggested that the mannose receptor may be of clinical interest for therapeutic interventions. For example, the mannose receptor is a potential target for the specific delivery of drugs to macrophages. [14,15] Furthermore, the receptor ligand t-PA is an enzyme that activates fibrinolysis and it has been shown that an increased plasma t-PA concentration present during thrombus formation reduces the thrombus formation in the rat. [16] Thus blockage of the mannose receptor-mediated plasma clearance of t-PA may be of interest in antithrombotic therapy. [17]

In vitro, the expressional state of the mannose receptor highly correlates with the functional state of the monocyte/macrophage. Macrophages express the mannose receptor whereas no expression is seen on monocytes. [2,18,19] High mannose receptor expression correlates with the antigen uptake capacity of dendritic cells [4] and increases foreign body-type giant cell formation.[6] Immunosuppressed or alternatively activated macrophages have a high mannose receptor expression, [2,4,19-22] whereas activated macrophages and dendritic cells have a low mannose receptor expression. [2,4,19,21-23] The mannose receptor may thus be a good marker for distinguishing between these macrophage phenotypes.

Although a number of studies have been performed on the expression of the mannose receptor under specific conditions *in vitro*, the *in vivo* expression of the mannose receptor has not been documented in detail. Recently we developed a monoclonal antibody (mAb) specific for the human mannose receptor (mAb 15-2). [24] In this report the expression of the mannose receptor under physiological and pathological conditions is evaluated by the use of mAb 15-2. Because it has been suggested that in most of the pathological conditions studied, macrophages are of crucial importance, we compared the mannose receptor expression to the expression of the commonly used pan-macrophage marker, the CD68 antigen, by the use of mAb KP-1. [25,26]

MATERIALS AND METHODS

Materials

Mouse monoclonal antibody KP-1 (isotype IgG_1) against the human CD68 antigen was purchased from Dako A/S (Glostrup, Denmark). Mouse monoclonal antibody mAb 15-2 (isotype IgG_1) has been raised against the human mannose receptor, isolated from placenta as described [24] (Gaubius laboratory, Leiden, The Netherlands). This mAb detects the 175 kDa mannose receptor in Western blot, [24] inhibits the binding of t-PA to the mannose receptor,[24] detects up- or downregulation of the macrophage mannose receptor expression by use of flow cytometry, [19] and can be used to detect mannose receptor expression in immunohistochemistry. [19]

Immunohistochemistry

Tissue obtained from autopsies and tissue biopsies obtained from patients for diagnostic purposes (Department of Pathology, Slotervaart Hospital, Amsterdam, The Netherlands) were snap frozen in liquid nitrogen. Diagnosis of tissues affected by disease was performed by pathologists using standard protocols. A modification of the immuno-alkaline-phosphatase method described by Li et al. [27] was used for tissue staining. Sections of 8 µm thickness were cut on a Reichert-Jung 2800 frigocut cryostat, transferred to poly-L-lysine-coated microscope slides, air dried and fixed in acetone (10 min at room temperature), rinsed (5 min) in phosphate-buffered saline (PBS), and incubated with primary antibody (1 µg/ml mAb 15-2, 1:2000 dilution of KP-1) for 30 min at room temperature. After rinsing in PBS (5 min), the sections were incubated with an alkaline-phosphatase-conjugated secondary antibody (rabbit anti-mouse, Dako A/S, Glostrup, Denmark) for 15 min at room temperature. After rinsing in PBS (5 min) the sections were incubated for 30 min at room temperature in the dark with naphtol-AS-MX-phosphate (Sigma Chemical Co., St.Louis, MO, USA) and New Fuchsine (Chroma Gezellschaft, Köngen, Germany) as a coupling salt to develop a red reaction product. Finally the sections were rinsed with aqua dest, counterstained with Mayers hematoxylin, and mounted in DePeX mounting medium (BDH, Poole, UK). In control sections no primary antibody was used. The identity of the cells was judged on the basis of their morphology and their location in the tissue.

RESULTS

Mannose receptor expression under physiological conditions

The expression of the mannose receptor and CD68 on cells in healthy tissues is summarized in Table 1, which includes some data that were recently published elsewhere. In all tissues only macrophages (including the perivascular glial cells and the synovial lining cells) were both CD68- and mannose receptor-positive. KP-1 and mAb 15-2 stained these macrophages with equal strength in healthy tissues. KP-1 and not mAb 15-2, stained monocytes, granulocytes, macrophages in thymus medulla, macrophages in the B cell areas of lymphoid tissue, Langerhans cells, brain microglia cells, and some endothelial and epithelial cells. In the liver and in the spleen mAb 15-2 and not KP-1, abundantly stained the sinusoidal endothelial cells (Fig 1A,B see appendix, page 140). In testis, the macrophages were both CD68- and mannose receptor-positive, the Leydig cells were only CD68-positive and the spermatids and spermatozoa were only mannose receptor-positive (Fig 1C,D see appendix, page 141).

TABLE 1 Tissue distribution of the mannose receptor and CD68 under physiological conditions.

Cryostat sections of human tissue were stained with mAb KP-1 or 15-2, and the staining patterns were compared with each other and to that of tissues stained without primary mAb. + positive cells, +(-) marker stained less cells than other marker, -(+) very few cells stain positive, - no staining observed, * mantle zone and germinal centre.

Tissue, location	cell type	KP-1	15-2
Bone marrow [19]			
	lymphocytes	-	-
	granulocytes	+	-
	monocytes	+	-
	megakaryocytes	-	-
	macrophages	+	+
Thymus [19]			
cortex	macrophages	+	+
medulla	macrophages	+	-(+)
	round cells	+	-
	endothelium	+	-
Lymph node [19]			
subcapsular sinus	macrophages	+	+
T cell area	macrophages	+	+(-)
B cell area*	macrophages	+	-(+)
medulla	macrophages	+	+
Spleen (Fig 1A)			
red pulp	sinusoidal cells	-	+
	macrophages	+	+
white pulp			
marginal zone	macrophages	+	+(-)
germinal centre	macrophages	+	-(+)
Liver [17]			
	sinusoidal cells	-	+
	Kupffer cells	+	+
	parenchymal cells	-	-
Kidney			
glomerulus	mesangial macrophages	+	+
proximal tubulus	epithelial cells	+	-
around tubuli	macrophages	+	+
Heart			
	endothelium	+	-
	heart muscle cells	-	-
	macrophages	+	+

Tissue, location	cell type	KP-1	15-2
Colon			
mucosa	macrophages	+	+
submucosa	macrophages	+	+
Brain			
	microglia cells	+	-
	perivascular glial cells	+	+
Placenta [24]			
	Hofbauer cells	+	+
	endothelial cells	+	-
Testis (Fig 1B)			
	macrophages	+	+
	Leydig cells	+	-
	spermatids	-	+
	spermatozoa	-	+
Skin [19]			
epidermis	Langerhans cells	+	-
dermis	macrophages	+	+
Lung			
	smooth muscle cells	-	-
	endothelium	+	-
	alveolar macrophages	+	+
	tissue macrophages	+	+
Trachea			
	macrophages	+	+
	smooth muscle cells	-	-
Synovium			
	synovial lining cells	+	+
	macrophages	+	+
Breast			
	macrophages	+	+

Mannose receptor expression under pathological conditions

As macrophages play an important role in granulomatous diseases, we studied the granulomatous diseases cutaneous tuberculoid leprosy, tuberculosis in the lung, and a reaction to polyethylene in the synovium (Table 2). The strength of the CD68 staining with KP-1 was not affected under these conditions (Fig 2A see appendix, page 144). In leprosy and tuberculosis, mAb 15-2 did not stain or only weakly stained the macrophages surrounding the (Langhans-type) giant cells (Fig 2B,C see appendix, page 144). In a synovium with macrophages reacting to polyethylene (foreign body reaction), mAb 15-2 strongly stained the macrophages surrounding the (foreign body-type) giant cells (Fig 2D see appendix, page 144). mAb 15-2 did not stain or only weakly stained both Langhans-type giant cells and foreign body-type giant cells (Fig 2B-D).

Healthy synovial lining cells expressed both the CD68 antigen and the mannose receptor (Table 1). In rheumatoid arthritis, hypertrophy and hyperplasia of the synovial lining cells was observed. Morphologically these cells appeared to be macrophages and the complete layer was CD68-positive. However, only a few cells of the hypertrophic and hyperplastic synovial lining cells were weakly mAb 15-2-positive (Fig 2E,F see appendix, page 145). In contrast to the stroma of normal breast, no mAb 15-2-positive macrophages were observed in the desmoplastic tumorstroma of breast carcinoma of both the ductal (Fig 2G,H see appendix, page 145) and the lobular type (not shown). Stromal macrophages in both normal breast and breast carcinoma were CD68-positive.

TABLE 2 Tissue distribution of the mannose receptor and CD68 under pathological conditions.

Cryostat sections of human tissue were stained with mAb KP-1 or 15-2, and the staining patterns were compared with each other and to that of tissues stained without primary mAb. + positive cells, ++ strong staining, -/+ weak staining, - no staining observed, (↑) mannose receptor upregulated and (↓) mannose receptor downregulated compared with physiological condition.

Disease, tissue, location	cell type	KP-1	15-2
Tuberculoid leprosy (Fig 2A,B)			
Skin			
epidermis	Langerhans cells	+	-
dermis	macrophages	+	+
granulomas	macrophages	+	-/+ (↓)
	Langhans-type giant cells	-/+	-
Tuberculosis (Fig 2C)			
Lung			
	alveolar macrophages	+	+
	tissue macrophages	+	-/+ (↓)
granulomas	pallisad macrophages	+	-/+
	Langhans-type giant cells	-/+	-/+
lymph aggregates	macrophages	+	-
Foreign body reaction (Fig 2D)			
Synovium			
	synovial lining cells	+	+
	stromal macrophages	+	++ (↑)
	foreign body-type giant cells	+	-/+
lymph aggregates	macrophages	+	-
Rheumatoid arthritis (Fig 2E,F)			
Synovium			
	Hypertrophic and hyperplastic synovial lining cells	+	-/+ (↓)
	stromal macrophages	+	+
Ductal/lobular carcinoma (Fig 2G,H)			
Breast			
normal stroma	macrophages	+	+
desmoplastic tumor stroma	macrophages	+	- (↓)

DISCUSSION

The expression and functions of the mannose receptor have been studied *in vitro*, however the *in vivo* expression of the mannose receptor is not documented in detail. We studied the expression of the human mannose receptor in human tissues under various conditions by immunohistochemistry using the recently-developed mAb 15-2. [19,24]

Many studies have suggested the involvement of the mannose receptor when a process was inhibited by ligands of the mannose receptor. However, there are other mannose-binding proteins that are able to bind mannose receptor ligands. [28-32] Thus inhibition of a process by mannose receptor ligands does not prove that the mannose receptor is involved. Because mAb 15-2 does not recognize these other mannose-binding proteins, [19] it is a useful tool for providing evidence for the involvement of the 175 kDa mannose receptor. This is illustrated by the observation that in the present study no mannose receptor was expressed by smooth muscle cells at all, while the mannose receptor has been reported to mediate mannose receptor ligand-induced proliferation by tracheal smooth muscle cells *in vitro*. [12,13] The discrepancy suggests that not the mannose receptor but another receptor is involved in this process. This other receptor might be the 180 kDa phospholipase A2 receptor; the receptor contains domains similar to the mannose receptor, [31] it binds mannose receptor ligands, and phospholipase A2 induces DNA synthesis in smooth muscle cells. [32,33] The 180 kDa phospholipase A2 receptor is not detected by mAb 15-2. [19]

Mannose receptor expression under physiological conditions.

The mannose receptor was expressed by resident (stromal) macrophages in nearly all vital organs under normal conditions. In contrast to CD68, the mannose receptor was not expressed by follicular dendritic cells in the germinal centres of lymphoid tissue (Fig 1A). As discussed previously [19] macrophages expressing both CD68 and the mannose receptor, represent non-activated macrophages or dendritic cells, while macrophages which express CD68 but not the mannose receptor, represent activated macrophages or dendritic cells. mAb 15-2 directed against the mannose receptor thus is an important tool in discriminating between non-activated macrophages/dendritic cells and activated macrophages/dendritic cells.

Besides the expression by macrophages, the mannose receptor was abundantly expressed by liver (see [17]) and splenic sinusoidal endothelial cells (Fig 1A), but not by endothelium in other organs. This suggests that the mannose receptor has a specific role in liver and spleen. As shown by plasma clearance and tissue distribution studies the uptake and degradation of mannose receptor ligands such as t-PA and mannosylated albumin from plasma is mainly mediated by the liver and not by the spleen. [8,34] Mannose receptor ligands have been used for the specific delivery of genes to mannose receptor-expressing cells in gene therapy. After injection of an expression plasmid conjugated to a mannose receptor ligand, a low expression of the gene was found in the liver whereas a high expression was found in the spleen. [15] This suggests that in contrast to the mannose receptor on liver sinusoidal endothelial cells, [35] the mannose receptor on splenic sinusoidal endothelial cells does not mediate efficient degradation of its ligands. Instead, the mannose receptor in the spleen appears to be involved in the homing of lymphocytes to the spleen, since mannan, a ligand for the mannose receptor, binds to the splenic sinusoidal endothelium, and has been shown to inhibit the splenic entry of lymphocytes *in vivo*. [9,10]

In brain tissue we found a single cell layer of mannose receptor-positive cells directly below the endothelium of blood vessels (these cells are the perivascular glial cells [36]), whereas the microglial cells were mannose receptor-negative. The perivascular glial cells are thought to be involved in the antigen presentation to encephalitogenic T cells in experimental autoimmune encephalomyelitis (EAE, an animal model for multiple sclerosis). [36] The mannose receptor has been shown to be involved in antigen presentation by young dendritic cells *in vitro*. [4,5] It is possible that the mannose receptor has a similar role in antigen presentation by perivascular glial cells. In addition, the mannose receptor may play a role in the blood-brain barrier by the uptake and degradation of mannose receptor ligands such as lysosomal enzymes [1,7] t-PA, [2,8] dopamine ß hydroxylase [37] and acetylcholinesterase. [38]

The mannose receptor was expressed on spermatids and spermatozoa (Fig 1B). Possibly the mannose receptor is involved in the fusion of sperm with the egg, as described for the involvement of the mannose receptor in the fusion of macrophages into foreign body-type giant cells, [6] but this remains to be shown. It has been shown that the mannose-albumin binding capacity of sperm cells correlates with the fertility of the sperm cells. [11] It is likely that the mannose receptor mediates the binding of mannose-albumin (a mannose receptor ligand) in the above-mentioned study, and that the mannose receptor expression thus may correlate with sperm fertility.

Mannose receptor expression under pathological conditions

In vitro, the expression of the mannose receptor is upregulated by interleukin-4, [20] interleukin-13, [21] dexamethasone [2,19,22] and prostaglandin E2. [23] On the other hand the mannose receptor activity is downregulated by lipopolysaccharide, [2,19] γ-interferon [21-23] or tumor necrosis factor-α, [4] substances known to activate macrophages. It has been suggested that interleukin-4 activates macrophages (alternative activation) in a different way from γ-interferon. [20] We have demonstrated previously that in flow cytometry, mAb 15-2 is able to detect mannose receptor downregulation on lipopolysaccharide-treated macrophages and mannose receptor upregulation on dexamethasone-treated macrophages *in vitro*. [19] Thus the intensity of staining with mAb 15-2 correlates with the amount of mannose receptor expressed by the cells. Here we demonstrated in human tissues that, compared with physiological conditions, under various pathological conditions the staining with mAb 15-2 was weaker or stronger on macrophages, whereas the staining with KP-1 was not altered. This indicates that the mannose receptor expression is down- or upregulated under these conditions *in vivo*.

In the granulomatous diseases studied, the mannose receptor was absent or only weakly expressed on both foreign body-type and Langhans-type giant cells. On the macrophages surrounding the foreign body-type giant cells, the mannose receptor appeared to be upregulated, whereas on the macrophages surrounding the Langhans-type giant cells the mannose receptor appeared to be downregulated (Fig 2A,B). This indicates that the macrophages in these granulomas are activated by different cytokines. The mannose receptor has been implicated as being involved in the fusion of macrophages into foreign body-type giant cells, since *in vitro* the formation can be inhibited by mannose receptor ligands. [6] Foreign body-type giant cell formation from macrophages is induced *in vitro* by interleukin-4 (T-helper 2 cytokine) treatment [6,39] that also upregulates the mannose receptor expression. [6,20,21] In contrast Langhans-type giant cell formation from macrophages is induced *in vitro* by γ-interferon (T-helper 1 cytokine) [39] which downregulates mannose receptor expression. [21-23] Thus the differences observed in mannose receptor expression in these granulomas possibly reflects the regulation by cytokines present in these human tissues *in vivo*.

In infectious granulomatous diseases such as tuberculoid leprosy and tuberculosis (Fig 2A,B) the mannose receptor appeared to be downregulated on the macrophages. Phagocytosis of virulent strains but not the less aggressive (attenuated) strains of *Mycobacterium tuberculosis* is mediated by the mannose receptor in addition to complement receptors *in vitro*.[40] Similarly, the parasites *Trypanosoma cruzi* and *Leishmani donovani* enter their host, the macrophage, via the mannose receptor.[3] The downregulation of the mannose receptor on macrophages may thus be an important defence mechanism against these pathogens.

In normal synovium the mannose receptor and the CD68 antigen were present in the macrophage-like cells, the synovial lining cells. In rheumatoid arthritis hyperplasia and hypertrophy of synovial lining cells was apparent (Fig 2E). This hyperplastic layer of cells was composed of CD68-positive and mannose receptor-negative (activated) macrophages. Because these activated macrophages are possibly involved in the tissue destruction seen in rheumatoid arthritis, it is of clinical importance to study their role in further detail. mAb 15-2 can be used to discriminate between activated and non-activated macrophages.

In the breast carcinomas studied here, the macrophages in the tumor stroma did not express the mannose receptor; this is in contrast to the macrophages in normal breast stroma (Fig 2D). This indicates that strong macrophage activation signals are present inside these tumors. Whether this activation is part of the defence mechanism of the body against the tumor or a defence mechanism of the tumor against the body (for example to avoid antigen presentation) remains to be shown. It would be interesting to study whether there is a correlation between macrophage mannose receptor expression and tumor malignancy.

Recently Uccini et al.[41] reported that the mannose receptor is expressed on Kaposi's sarcoma cells and that these cells are possibly related to splenic sinusoidal endothelial cells. As mAb 15-2 is capable of detecting mannose receptor-expressing cells by use of flow cytometry,[19] mAb 15-2 may be an useful tool in detecting (precursors of) these sarcoma cells in peripheral blood for diagnostic purposes, but this remains to be shown.

We conclude that only a few cell types express the mannose receptor in man and that, as observed *in vitro*, the mannose receptor may be up- or down- regulated on macrophages in human tissues under pathological conditions *in vivo*. The anti-mannose receptor mAb 15-2 is a specific marker for non-activated macrophages, liver and splenic sinusoidal endothelial cells, and is possibly a marker for fertile sperm.

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CHAPTER 6

Lysine-based cluster mannosides that inhibit ligand binding to the human mannose receptor at nanomolar concentration.

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SUMMARY

In search of synthetic high affinity ligands for the mannose receptor, we synthesized a series of lysine-based oligomannosides containing two (M_2L) to six (M_6L_5) terminal α-D-mannose groups that are connected with the backbone by flexible elongated spacers (16Å).

The synthesized cluster mannosides were all able to displace binding of biotinylated ribonuclease B and tissue-type plasminogen activator to isolated human mannose receptor. The affinity of these cluster mannosides for the mannose receptor was continuously enhanced from 18-23 μM to 0.5-2.6 nM with mannose valencies increasing from two to six. On average, expansion of the cluster mannoside with an additional α-D-mannose group resulted in a 10-fold increase in its affinity for the mannose receptor. M_3L_2 to M_6L_5 displayed negative cooperative inhibition of ligand binding to the mannose receptor, suggesting that binding of these mannosides involves multiple binding sites.

The nanomolar affinity of the most potent ligand, the hexamannoside M_6L_5 makes it the most potent synthetic cluster mannoside for the mannose receptor yet developed. As a result of its high affinity and accessible synthesis, M_6L_5 not only is a powerful tool to study the mechanism of ligand binding by the mannose receptor, but it is also a promising targeting device to accomplish cell-specific delivery of genes and drugs to liver endothelial cells or macrophages in bone marrow, lungs, spleen and atherosclerotic plaques.

INTRODUCTION

The mannose receptor is a 175-kDa membrane-associated protein that is localized on sinusoidal liver cells, peripheral and bone marrow macrophages, and dendritic cells. [1-4] It recognizes and internalizes mannosylated polysaccharides from pathological microorganisms, [5] tumour cells, [6] and yeast cells [7] and glycoproteins like type-I procollagen, [8] tissue-type plasminogen activator, [9] or various lysosomal enzymes. [10] As such, the mannose receptor participates in the nonimmune host-defence system. In addition, the macrophage receptor is implicated in major histocompatability complex-mediated antigen presentation by dendritic cells. [11]

The cDNA of the mannose receptor has been sequenced by Taylor *et al.* [12] and codes for five types of domains [13]: an N-terminal cysteine-rich domain, a transmembrane domain, a fibronectin type II-like domain, a domain composed of eight strongly homologous repeats (the so-called carbohydrate recognition domains or CRDs) and a C-terminal cytoplasmic tail. Taylor and Drickamer have established that the CRDs are involved in ligand binding. [13,14] Recent structure-function studies of recombinant truncated forms of the mannose receptor provided new insight into the mechanism of ligand binding by the mannose receptor. [13,14] On basis of these results, it was proposed that CRD4 is the only CRD to display a monosaccharide specificity characteristic for the mannose receptor. [15] CRD4 and CRD5 appear to be required for high affinity binding of high mannose-type glycoproteins and mannosylated bovine serum albumin (BSA). By contrast, at least five consecutive CRDs are needed for avid binding of highly mannosylated polysaccharides like mannan. [14]

Since each CRD embeds only a single mannose binding site, this implies that the mannose receptor may accommodate five to eight appropriately configured terminal α-D-mannose residues. Previous studies by Hoppe *et al.* [16] and Janssen *et al.* [17] established that the affinity of mannosylated albumin for

the mannose receptor indeed correlated with the extent of mannosylation. Surprisingly, derivatization with more than 22-24 mannose groups was required for high affinity recognition by the mannose receptor. This is considerably higher than the maximum number of CRDs thought to participate in ligand binding. On one hand the above finding may reflect a purely entropic phenomenon, caused by an increased chance of α-D-mannose groups to be adequately configured with respect to each other. On the other hand, it may suggest that multiple receptor molecules cooperate in the ligand binding process.

To address this we have evaluated the effect of mannose valency on the affinity for the mannose receptor in close detail using a series of homologous cluster mannosides that possess two to six terminal α-D-mannose groups. This study shows that recognition of low molecular weight mannosides by the mannose receptor is consistently, and not in a stepwise manner, enhanced with valencies increasing from two to six and thus provides new information on the process of ligand binding by the mannose receptor.

MATERIALS AND METHODS

Materials

$Na^{125}I$ in 0.1 M NaOH (13.5 mCi/μg) was purchased from Amersham (Buckinghamshire, United Kingdom). BSA (fraction V, delipidated), collagenase (type I), p-nitro-phenolphosphate, p-aminophenyl-α-D-mannopyranoside, and ribonuclease B (bovine pancreas) were purchased from Sigma. L-Lysine-HCl; L-lysyl-L-lysine,2 HCl. 0.5 H_2O; di-(L-lysyl)-L-lysine,3AcOH; tri-(L-lysyl)-L-lysine, 4AcOH; and tetra-(L-lysyl)-L-lysine, 5AcOH were all obtained from Bachem Feinchemikalien AG (Bubendorf, Switzerland). Thiophosgene was obtained from Aldrich Chemie (Bornem, Belgium). N,N-Dimethylformamide obtained from Merck (Hohenbrunn, Germany) was refluxed for 4 h with CaH_2 (5 g/liter) and destilled under reduced pressure. Streptavidin-alkaline-phosphatase conjugate was from Amersham. Tissue-type plasminogen activator (t-PA) from a recombinant human melanoma cell culture was provided by Dr. J. Verheijen (Gaubius Laboratory, TNO-PG, Leiden, The Netherlands).

Chromatography

Thin layer chromatography was performed using silica F_{254} preformed layers (0.1 mm) on a plastic backing (Schleicher & Schuell DC-F1500) and MeOH (A), acetonitrile-H_2O (80:20, v/v) (B), or MeOH-CH_2Cl (80:20, v/v) (C) as eluent. Carbohydrates were visualized after spraying with 20% H_2SO_4 in MeOH and subsequent heating at 140-160°C. Amine- or amide-containing compounds were visualized after spraying with ninhydrin and subsequent heating at 140°C, while compounds containing aromatic group were visualized by UV shadowing (254 nm). Preparative column chromatography was performed using Kieselgel 60 (Merck, Hohenbrunn, Germany).

Instrumental analysis

NMR spectra were recorded at 300 MHz (^1H) with a Bruker WM300 spectrometer operating in the Fourier Transform mode (FT). Chemical shifts are denoted in ppm (δ) relative to tetramethylsilane as internal standard. Mass spectra were obtained using a Finnigan MAT 900 mass spectrometer, using electrospray (MeOH/H_2O (80/20) + 1% HAc) as the ionization technique. Positive ions were visualized after data processing using the HMR BSCAN UP profile.

Synthesis

4-(α-D-Mannopyranosyloxy)phenylisothiocyanate (**1b**) - Compound **1a** (scheme 1) was converted into the phenyl isothiocyanate derivative (**1b**) as described by Monsigny et al. [18] and Kataoka et al.[19] In short, a mixture of compound **1a** (244 mg, 0.9 mmol) and thiophosgene (0.52 mL, 5.1 mmol) in ethanol/H_2O (80:20, v/v; 50 mL) was stirred for 2 h at room temperature. The excess of thiophosgene was removed by perspiration with N_2 for 1 h at room temperature. After concentration of the solution under reduced pressure, the residue was dissolved in a small volume of distilled water (1-2 ml), and NaOH (1.0 M) was added to pH 6.0. The solution was concentrated, and the residue was chromatographed over a Kieselgel 60 column (40 ml) using CH_2Cl_2/MeOH (80:20, v/v) as eluent. Fractions containing product **1b** were pooled and lyophilized to yield 288 mg of a white crystaline powder (0.916 mmol; 102%); R_F: 0.75 (B), 0.67 (C). Mass: 313.1 (M^+ calculated: 313.32); ^{13}C-NMR Attached Proton Test (APT):
161.3 (C_1-phenyl), 138.5 (C_4-phenyl), 127.8 (C_2,6 -phenyl), 118.9 (C_3,5 -phenyl), 101.1 (C_1 - Man, α-configuration), 76.9 (C_5-Man), 73.6 (C_2 -Man), 72.1 (C_3 -Man), 70.0 (C_4 -Man), 63.3 (C_6 -Man).

Mannosylated oligolysines

Mannosylated (oligo)lysines, *i.e.* M_2L (**2a**), M_3L_2 (**2b**), M_4L_3 (**2c**), M_5L_4 (**2d**) and M_6L_5 (**2e**) were synthesized according to the procedure of Jansen *et al.* [17] with slight modifications. In general, compound **1b** (0.10 mmol; 32 mg) and (oligo)lysine (0.83 molar equivalents on the basis of the amino group content) were dissolved in a mixture of 0.1 M sodium hydrogen carbonate (pH 8.5) and N,N-dimethylformamide (50:50, v/v; 4 ml), and the solution was stirred for 18h in the dark at room temperature. The progression of the reaction was monitored by TLC. In case all of compound **1b** had reacted, while the reaction was not yet complete, an additional amount of **1b** (0.4 molar equivalents) was added, and the reaction mixture was incubated for another 8 h. Subsequently, the mixture was concentrated *in vacuo*, and the crude products **2a-e** were chromatographed over a Kieselgel 60 column using acetronitrile/H_2O (80:20, v/v) as eluent. Fractions containing product **2a-e** were pooled and lyophilized to yield a whitish powder.

*N^{ε},N^{ε}-Bis[N-(p-(α-D-mannopyranosyloxy)anilino)thiocarbamyl]-L-lysine (M_2L; **2a**)*

R_F= 0.31(A); yield: 5.2 µmol (12.5%); Mass (M+Na$^+$): 794.6 (M$^+$ calculated: 771.5); ^{1}H-NMR (D_2O/ CD_3OD): δ 1.27 (q, br, 2H, CH_2-γ), 1.63 (q, br, 2H, CH_2-δ), 1.90 (q, br, 2H, CH_2-β), 3.50 (t, br, 2H, CH_2-ε), 3.75-4.13 (m, 11H, Man H-1 to H-5 and CH-α); 4.04 (d, br, Man H-1(α), *J*=3.2 ppm); 5.61 (dd, 4H, Man H-6 and H-6'), 7.17 (m, 8H, phenyl-CH).

*N^{ε}-[N^{ε},N^{ε}-Bis[N-(p-(α-D-mannopyranosyloxy)anilino)thiocarbamyl]-L-lysyl]-N^{ε}-[N-(p-(α-D-mannopyranosyloxy)anilino) thiocarbamyl]-L-lysine (M_3L_2: **2b**).* R_F (A)= 0.20; yield: 11.1 µmol (40%); Mass (M+Na$^+$): 1235.8 (M$^+$ calculated: 1212.8); ^{1}H-NMR (D_2O/ CD_3OD): δ 1.33 (q, 4H, CH_2-γ), 1.54 (q, br, 4H, CH_2-δ), 1.76 (q, br, 4H, CH_2-β), 3.39 (t, br, 4H, CH_2-ε), 3.73-4.19 (m, 17H, Man H-1 to H-5 and CH-α); 4.06 (d, br, Man H-1(α), *J*=3.1 ppm); 5.57 (dd, 6H, Man H-6 and H-6'), 7.22 (m, 12H, phenyl-CH).

*N^{ε}-[N^{ε}-[N^{ε},N^{ε}-Bis[N-(p-(α-D-mannopyranosyloxy)anilino)thiocarbamyl]-L-Lysyl]-N^{ε}-[N-(p-(α-D-mannopyranosyloxy)anilino)-thiocarbamyl]-L-lysyl]-N^{ε}-[N-(p-(α-D-mannopyranosyloxy)anilino)thiocarbamyl]-L-lysine (M_4L_3: **2c**).* R_F (A): 0.11; yield: 7.0 µmol (34%); Mass (M+Na$^+$): 1676.1 (M$^+$ calculated 1654.1); ^{1}H-NMR (D_2O/CD_3OD): δ 1.26 (q, 6H, CH_2-γ), 1.57 (q, br, 6H, CH_2-δ), 1.90 (q, 6H, CH_2-β), 3.31 (t, br, 6H, CH_2-ε), 3.68-4.26 (m, 23H, Man H-1 to H-5 and CH-α), 3.97 (d, br, Man H-1(α), *J*=2.9 ppm); 5.52 (dd, 8H, Man H-6 and H-6'), 7.05 (m, 16H, phenyl-CH)

*N^{ε}-[N^{ε}-[N^{ε}-[N^{ε},N^{ε}-Bis[N-(p-(α-D-mannopyranosyloxy)anilino)thiocarbamyl]-L-lysyl]-N^{ε}-[N-(p-(α-D-mannopyranosyloxy)anilino) thiocarbamyl]-L-lysyl]-N^{ε}-[N-(p-(α-D-mannopyranosyloxy)anilino)thiocarbamyl]-L-lysyl]-N^{ε}-[N-(p-(α-D-mannopyranosyl-oxy)anilino) thiocarbamyl]-L-lysine (M_5L_4: **2d**).* R_F (A): 0.05; Yield: 11.5 µmol (50%); Mass (½.M^{2+}+Na$^+$):1072.6 (M $^+$ calculated: 2095.4); ^{1} H - NMR (D_2O/CD_3OD): δ 1.38 (q, br, 8H, CH_2-γ), 1.75 (q, br, 8H, CH_2-δ), 1.88 (q, 8H, CH_2-β), 3.34 (t, br, 8H, CH $_2$-ε), 3.66-4.36 (m, 29H, Man H-1 to H-5 and CH-α); 3.95 (d, br, Man H-1(α), *J*=2.5 ppm); 5.47 (dd, 10H, Man H-6 and H-6'), 7.05 (m, 20H, phenyl-CH)

*N^{ε}-[N^{ε}-[N^{ε}-[N^{ε}-[N^{ε},N^{ε}-Bis[N-(p-(α-D-mannopyranosyloxy)anilino)thiocarbamyl]-L-lysyl]-N^{ε}-[N-(p-(α-D-mannopyranosyloxy)anilino) thiocarbamyl]-L-lysyl]-N^{ε}-[N-(p-(α-D-manno-pyranosyloxy)anilino)thiocarbamyl]-L-lysyl]-N^{ε}-[N-(p-(α-D-manno-pyranosyloxy) anilino)t-hio-carbamyl]-L-lysyl]-N^{ε}-[N-(p-(α-D-mannopyranosyl-oxy)-anilino)thiocarbamyl]-L-lysine (M_6L_5; **2e**).* R_F (A): 0.05; yield: 11.5 µmol (83%); Mass (½*M^{2+} +Na$^+$): 1291.3 (M $^+$ calculated: 2536.7); ^{1}H-NMR (D_2O/CD_3OD): δ 1.32 (q, br, 8H, CH_2-γ), 1.52 (q, br, 8H, CH_2-δ), 1.74 (q, 8H, CH_2-β), 3.43 (t, br, 8H, CH_2-ε), 3.65-4.28 (m, 29H, Man H-2 to H-5 and CH-α), 3.91 (d, br, Man H-1(α), *J*=2.7 ppm); 4.92 (dd, 10H, Man H-6 and H-6'), 7.06-7.10 (m, 20H, phenyl-CH).

Biotinylation or radio-iodination of t-PA and ribonuclease B

Ribonuclease B or t-PA were dialyzed against 0.1 M $NaHCO_3$, pH 8.5, and subsequently incubated for 3h at room temperature with N-hydroxysuccinimide-activated biotin (Zymed Laboratories Inc., South San Francisco, CA) at a ratio of 1 mol of protein to 6 mol of N-hydroxysuccinimide-activated biotin for ribonuclease B, and 200 mol of of N-hydroxysuccinimide-activated biotin for t-PA, respectively. After reaction, the protein was dialyzed against 20 mM Tris/HCl buffer, pH 7.4, containing 150 mM NaCl, 5 mM $CaCl_2$ and 0.5% (v/v) Tween-80. t-PA was radioiodinated by the IODO-GEN method as described, and a specific radioactivity of 3500-5000 cpm/ng of protein was obtained. [20]

Isolation of endothelial liver cells.

Rat endothelial liver cells were isolated by a collagenase perfusion protocol at 37°C as described previously. [21] Liver cells were separated by differential centrifugation and endothelial cells were subsequently purified by counterflow centrifugation. The purity of endothelial cells as monitored by peroxidase staining was at least 95%. Viability of the cells used for *in vitro* experiments as judged by 0.2% trypan blue exclusion.

Isolation of human mannose receptor

Human mannose receptor was isolated from human placenta after solubilisation with Triton X-100 and subsequent affinity chromatography over mannosylated albumin-sepharose according to Otter *et al.* [22]

Mannose receptor binding assay

Displacement studies of binding of biotinylated ribonuclease B and biotinylated t-PA to isolated human mannose receptor were performed essentially as described by Otter *et al.*[9] Activated PVC multiwell plates (Flow Laboratories) were coated overnight at 4°C with purified mannose receptor (±15 ng/well) in coating buffer (100 µl; pH 7.4), containing 20 mM Tris/HCl, 150 mM NaCl, 5 mM $CaCl_2$. After washing, the wells were incubated for 30 min at 20°C with assay buffer (coating buffer supplemented with 0.5% Tween 80 and 0.1% BSA) (125 µl) to minimize aspecific ligand binding. Then the wells were washed and preincubated for 30 min at 20°C with a solution of α-D-mannose (10 µM-100 mM) or one of the cluster mannosides (1 nM-2 mM) in assay buffer (100 µl). Biotinylated ribonuclease B or biotinylated t-PA in assay buffer was added to a final concentration of 580 and 1.3 nM, respectively, and the mixture was incubated for 2 h at 20°C. After a washing step, the wells were incubated for 1 h at room temperature with streptavidin-alkaline phosphatase conjugate (1:1000 dilution in assay buffer; 100 µl). p-Nitrophenolphosphate (1 mg/ml; 100 µl in a 100 mM diethanolamine, 5 mM $MgCl_2$-buffer, pH 9.5) was added after thorough rinsing of the wells. The plate was incubated for 4 h at 25 °C during which the ΔA405/h was determined as a measure of ligand binding. Uncoated wells were used as a control for aspecific adherence of biotinylated ribonuclease B or biotinylated t-PA to the wells.

Competition studies of ^{125}I-t-PA binding to endothelial liver cells

Competition studies of ^{125}I-t-PA binding to endothelial liver cells were performed as described before. [23] Endothelial cells (2x10 ; 150 µg of cell protein), in Dulbecco's modified Eagle's medium (DMEM, 0.5 ml), containing 2% (w/v) BSA and ^{125}I-t-PA (1 nM), were incubated for 2 h at 4°C with a variable concentration of unlabeled biotinylated t-PA (bio-t-PA), biotinylated Ribo-B (bio-Ribo B) or M_6L_5 ranging from 0.1 nM to 20 µM. Following incubation, cells were washed twice with DMEM+0.2% BSA and once with DMEM, and the cell-associated radioactivity was counted. Nonspecific binding was defined as ^{125}I-t-PA binding in the presence of 100 mM α-D-mannose. Cell-bound radioactivity was determined using a Packard γ-counter and corrected for protein content.

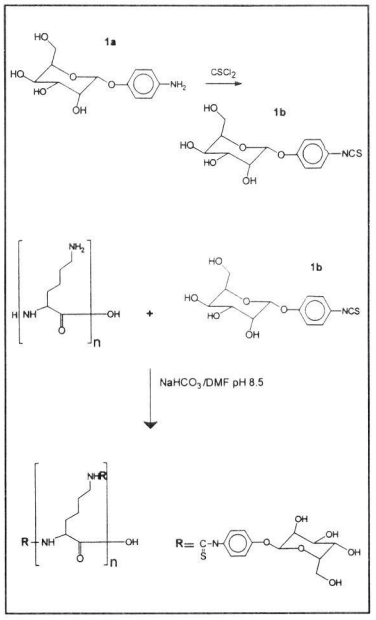
Data analysis

To calculate the IC_{50} values and Hill coefficients (n_H), the displacement binding data were analyzed according to the following binding model: percentage of specific binding = $100/(1+ ((\text{displacer})/IC_{50}^{nH})$ using a computerized nonlinear fitting program (Prism, ISI software; Ref. 24). As a measure of the actual affinity for the mannose receptor, apparent inhibition constants (dissociation constants) were calculated from the IC_{50} values using the equation $K_{i\,(app)} = IC_{50}/(1+(\text{Ligand})/K_d)$, and using K_d values for bio-tinylated ribonuclease B and t-PA obtained from the saturation binding studies (550 and 1.66 nM, respectively). The irregular competition curves of α-D-mannose were also fitted according to a two-site mixed stimulation/inhibition model (percentage specific binding= (100 + percentage maximal stimulation x (1 -1/(1+(displacer) / SC_{50})))/ (1+(displacer)/ IC_{50}), SC_{50} being the concentration at which half maximal stimulation of ligand binding to the mannose receptor is attained. The significance of the differences between means was tested by unpaired two-way Student's-t test.

RESULTS

Synthesis of the cluster mannosides

The cluster mannosides were prepared by a two-step synthetic procedure. First, 4-aminophenyl-α-D-mannopyranoside (**1a**) was quantitatively converted into 4-(α-D-mannopyranosyloxy)phenyl-isothiocyanate using thiophosgene (**1b**). Second, the activated α-D-mannopyranoside **1b** was reacted with a series of oligolysines, *i.e.* mono-, di-, tri-, tetra- and pentalysine. Progression of the coupling was monitored on TLC by ninhydrin staining. The reaction was considered to be complete when no significant ninhydrin-positive spots, indicative of unreacted amino groups, could be detected. Subsequent chromatography of the crude reaction mixture over Kieselgel 60 and lyophilization afforded product **2a-e** as white crystalline powders at yields ranging from 34 to 83%. TLC analysis (UV, ninhydrin, sulphuric acid detection) confirmed that the isolated products **2a-e** were fully mannosylated, did not contain any free amino groups and were apparently pure. ^{1}H-NMR and mass spectrometry of the isolated products **2a-2e** were in agreement with the chemical structure of the anticipated products M_2L, M_3L_2, M_4L_3, M_5L_4 and M_6L_5 (for chemical structures see Fig 1).



Scheme 1 **Reaction scheme of the synthesis of clustermannosides 2a to 2e.** **2a**, n=1; **2b**, n=2, **2c**, n=3; **2d**, n=4; **2e**, n=5.

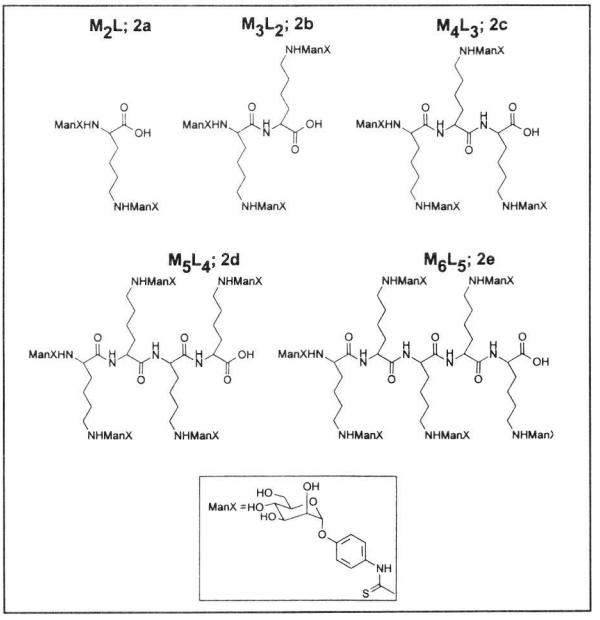


Fig 1 **Chemical structures of the cluster mannosides 2a to 2e.**

Competition binding studies

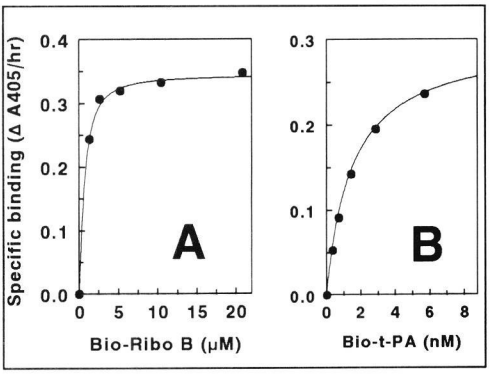
First we have performed saturation studies of bio-Ribo B and bio-t-PA binding to the isolated mannose receptor in the enzyme-linked receptor sorbent assay described by Otter et al. [9] and Barrett-Bergshoeff et al. [25] (Fig 2). Bio-Ribo B and bio-t-PA binding appears to be saturable (0.354 ± 0.006 ΔA/hr and 0.305 ± 0.003 ΔA/hr, respectively) and obeyed classical law of mass action kinetics. The maximal binding capacity of bio-Ribo B and bio-t-PA were comparable. Analysis of the binding curves gave dissociation constants of 550 ± 70 nM for bio-Ribo B and 1.66 ± 0.05 nM for bio-t-PA. Hill coefficients were calculated to be close to unity (1.16 ± 0.43 and 1.13 ± 0.03, respectively), indicative of ligand binding to a single binding site.

Subsequently, competition studies of bio-Ribo B binding were performed for the synthesized mannosides. As can be seen from Fig 3A, all of the tested mannosides were able to completely inhibit the specific binding of bio-Ribo B to the isolated human mannose receptor. The potency of the compounds to inhibit bio-Ribo B binding was significantly increased with increasing mannose valency. The apparent inhibition constant $K_{i\,(app)}$ of α-D-mannose was at least 10^6 -fold higher than that of the hexamannoside M_6L_5, the most potent ligand in this study (2.7 mM and 2.6 nM, respectively, Table 1). Even the dimannoside M_2L possessed a considerably higher affinity ($K_{i\,(app)}$ =17.5 μM) than α-D-mannose.

Fig 2

Saturation curve of bio-Ribo-B binding (A) and bio-t-PA (B) binding to isolated human mannose receptor in a receptor sorbent assay.

Multiwells, coated with isolated human mannose receptor, were incubated for 2h at 20°C with a variable concentration of biotinylated ribonuclease B or biotinylated t-PA. After incubation, ligand binding was determined as described under "Materials and Methods". Specific binding of bio-Ribo B and bio-t-PA is plotted as ΔA/h and is corrected for aspecific binding determined in the presence of uncoated wells. The saturation binding curves were fitted using computerized nonlinear regression procedures.



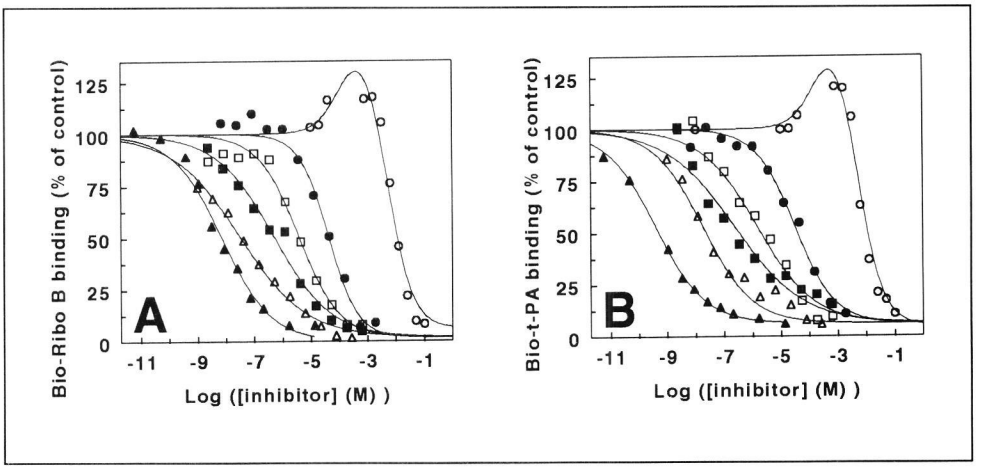


Fig 3 Competition studies of binding of biotinylated ribonuclease B (A) or biotinylated t-PA (B) to the isolated human mannose receptor by the following mannosides: α-D-mannose (○); M_2L (●); M_3L_2 (□); M_4L_3 (■); M_5L_4 (Δ) and M_6L_5 (▲).

Multiwells, coated with isolated human mannose receptor, were incubated for 2h at 20°C with a fixed concentration of biotinylated ribonuclease B (580 nM) or biotinylated t-PA (1.3 nM) in the absence or presence of displacer, at concentrations ranging from 10^{-10} to 10^{-1} M. After incubation, ligand binding was determined as described under "Materials and Methods". Total binding of bio-Ribo B and bio-t-PA is plotted as percentage of the control (without displacer) *versus* the log of the displacer concentration (in M). The inhibition curves for α-D-mannose, M_2L, M_3L_2, M_4L_3, M_5L_4, and M_6L_5 were fitted using computerized nonlinear regression procedures.

To verify that the cluster mannosides inhibited bio-Ribo B binding in a competitive fashion by blocking the mannose binding site on the mannose receptor rather than by directly interfering with bio-Ribo B itself, competition studies were also performed using another ligand for the human mannose receptor, biotinylated tissue-type plasminogen activator (bio-t-PA) [22] (Fig3B). In data not shown here it was demonstrated that bio-t-PA binding to the mannose receptor was fully inhibited in a competitive fashion by ribonuclease B, indicating that both ligands interact with the same binding site on the mannose receptor.

Tabel 1 Affinity of the cluster mannosides as determined from competition studies of binding of biotinylated ribonuclease B or t-PA to the isolated mannose receptor.

-Log(IC_{50}) values (± S.D.) and $K_{i(app)}$ values were calculated from the displacement curves using nonlinear regression analysis.

Compound	Bio-Ribo B		Bio-t-PA	
	-Log(IC_{50}) (±S.D.)	$K_{i(app)}$ (nM)	-Log(IC_{50}) (±S.D.)	($K_{i(app)}$) (nM)
α-D-mannose	2.00 (±0.04)	2,700,000	1.98 (±0.04)	2,800,000
M_2L	4.49 (±0.07)	17,500	4.38 (±0.07)	22,600
M_3L_2	5.76 (±0.10)	950	5.43 (±0.09)	2020
M_4L_3	6.61 (±0.15)	135	6.30 (±0.09)	270
M_5L_4	7.56 (±0.08)	15.1	7.78 (±0.07)	8.9
M_6L_5	8.33 (±0.09)	2.6	9.05 (±0.09)	0.5

The bio-t-PA competition curves clearly demonstrated that the potency of the cluster mannosides to inhibit bio-t-PA binding was analogously increased with increasing mannose valency. ($K_{i(app)}$ values calculated from the bio-t-PA competition curves ranged from 2.8 mM for α-D-mannose to 8.9 nM for M_5L_4 and 0.5 nM for M_6L_5. This is illustrated in Fig 4 in which -log (IC_{50}) values of the mannosides from the bio-Ribo B assay are plotted against those from the bio-t-PA assay. Clearly, the affinities in both assays correlate excellently (correlation coefficient 0.984; $P < 0.0001$; slope 1.11 ± 0.07).

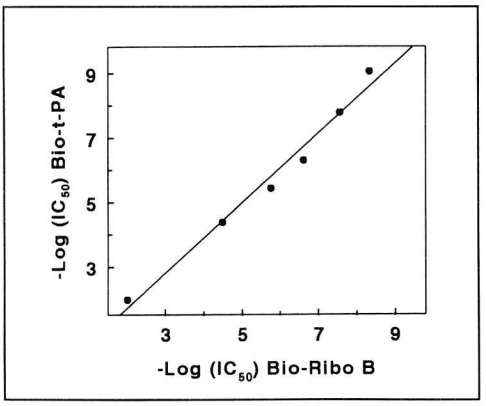


Fig 4 Correlation between the potency of the mannosides to inhibit bio-t-PA and ribonuclease B binding to the isolated mannose receptor.

The inhibitory potencies of the cluster mannosides are derived from the data in Table 1 and are expressed as -log(IC_{50}) ± S.D. Invisible error bars indicate errors smaller than the *symbol* size. r= 0.984, slope= 1.11 ± 0.07.

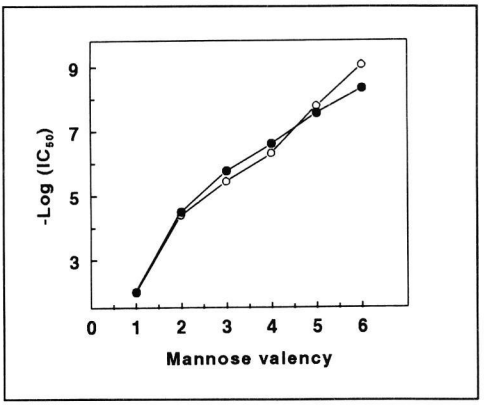


Fig 5 Effect of the mannose valency on the potency of cluster mannosides to inhibit binding of biotinylated ribonuclease B (●) or t-PA (○) to isolated human mannose receptor.

Inhibitory potencies of the cluster mannosides are derived from the data in Table 1 and are expressed as -log(IC_{50}) ± S.D. Invisible error bars indicate errors smaller than the *symbol* size.

Fig 5 shows that there is only a minor tendency of the -log (IC_{50}) values to level off at high mannose valencies, suggesting that the cluster mannosides did not yet reach the theoretical maximum affinity. Moreover, the increase in affinity is steady and intermediate plateau values for the cluster mannosides were not observed.

To calibrate the receptor sorbent assay, competition studies of ^{125}I-t-PA binding to endothelial liver cells were performed for M_6L_5, bio-t-PA and bio-Ribo B. All three tested ligands were able to inhibit ^{125}I-t-PA binding (Fig 6) at inhibition constants that were congruent to those found in the receptor sorbent assay ($K_{i\,(app)}$ = 1.7 ± 0.2 nM, 3.4 ± 0.4 nM, and 190 ± 70 nM, respectively). This further substantiates the validity of $K_{i\,(app)}$ values derived from this receptor sorbent assay.

Interestingly, displacement of bio-Ribo B and bio-t-PA binding to the human mannose receptor by the mannosides was not monophasic, as judged from the low Hill coefficients of M_3L_2, M_4L_3, M_5L_4, and M_6L_5 (n_H= 0.4 - 0.5) (Fig 7). In contrast, saturation curves of bio-Ribo B and bio-t-PA binding to isolated mannose receptor proceeded in a purely competitive fashion (n_H = 1.16 ± 0.43 and 1.17 ± 0.02, respectively). In both assays, the bivalent mannoside M_2L exhibited an intermediate Hill coefficient of 0.63 ± 0.05 (bio-Ribo B) and 0.85 ± 0.1 (bio-t-PA). The Hill coefficients of both ligand binding assays correlated significantly (r^2= 0.990, slope 1.006 ± 0.05; P < 0.0002). Competition curves of α-D-mannose were clearly irregular. α-D-mannose appeared to stimulate ligand binding to the mannose receptor by ± 25% at concentrations ranging from 30 to 300 µM. At higher concentrations, α-D-mannose inhibited binding in a positive cooperative fashion with Hill coefficients of 2.1 ± 0.5 (bio-Ribo B) and 2.2 ± 0.6 (bio-t-PA).

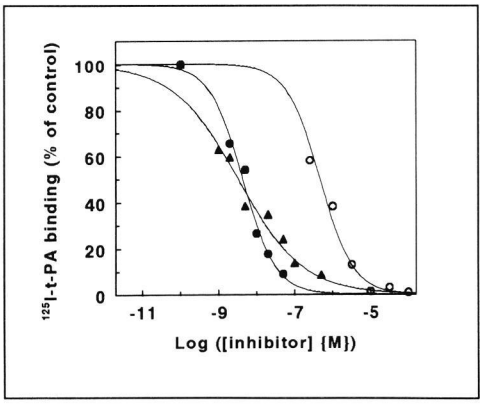


Fig 6 Competition studies of ^{125}I-t-PA binding to endothelial liver cells by bio-Ribo B (○); bio-t-PA (●); and M_6L_5 (▲).

Rat endothelial liver cells (2 x 10^6; 150 µg of cell protein), in DMEM (0.5 ml), containing 2% (w/v) BSA and ^{125}I-t-PA (1 nM), were incubated for 2 h at 4°C with a variable concentration of displacer. Following incubation, cells were washed twice with DMEM, 0.2% BSA and once with DMEM, and cell-associated radioactivity was counted. Nonspecific binding is defined as ^{125}I-t-PA binding in the presence of 100 mM α-D-mannose.

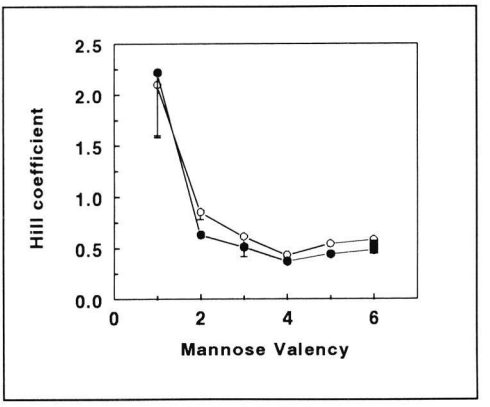


Fig 7 Effect of the valency of the (cluster) mannosides on the Hill coefficient of bio-Ribo B (●), bio-t-PA (○) and ^{125}I-t-PA (■).

Hill coefficients were calculated from the displacement curves in Figs. 3, 4 by nonlinear regression analysis as described under "Materials and Methods".

Competition studies of 4-aminophenyl α-D-mannopyranoside also gave a 33% stimulation combined with a positive cooperative inhibition (n_H=1.96 ± 0.143; data not shown). Since the stimulatory effect and the high apparent Hill coefficients were found in both receptor sorbent assays and in the endothelial cell binding assay (M_6L_3), we assume that it did not involve an artefact. Nonlinear regression analysis according to a two-site mixed stimulation/inhibition model showed that α-D-mannose induced a maximal stimulation of bio-Ribo B and bio-t-PA binding of 53% and 58%, respectively, at low concentrations. Half maximal stimulation of ligand binding to the mannose receptor was attained at 250 μM (-log(SC_{50}) = 3.6 ± 0.2) and 400 μM (-log(SC_{50}) = 3.4 ± 0.2), respectively. Full inhibition of ligand binding was attained at a -log(IC_{50}) of 2.00 ± 0.05 and 1.98 ± 0.04, resp. The above mixed stimulation/inhibition model excellently predicted the observed steep inhibition curve for α-D-mannose.

DISCUSSION

In this study we have investigated the effect of the mannose valency of a cluster mannoside on its affinity for the mannose receptor. The ability of cluster mannosides to inhibit the binding of Ribo B, an established mannose receptor ligand, [1] to the isolated human mannose receptor was determined in a receptor sorbent binding assay. Previous studies by Otter *et al.* [9,25] have established that this assay provides a reliable estimate of the affinity of monosaccharides, proteins with high mannose-type glycosides, and mannosylated neoglycoproteins for the mannose receptor. The validity of the receptor sorbent assay was further documented by the finding that the K_d for bio-Ribo-B (550 ± 70 nM) and bio-t-PA (1.66 ± 0.05 nM) in the receptor sorbent assay paralleled K_i values derived from competition studies of ^{125}I-t-PA binding to endothelial liver cells (190 ± 70 μM, and 3.4 ± 0.4 nM, respectively).

The competition studies showed that all mannosides were able to inhibit bio-Ribo B binding to the isolated mannose receptor. The affinity of the synthesized mannosides was strongly and consistently enhanced with increasing mannose valency. Since the apparent inhibition constants $K_{i\,(app)}$ in the bio-Ribo B and bio-t-PA binding assays were essentially equal and ribonuclease B inhibits bio-t-PA binding in a competitive fashion, we may assume that inhibition of bio-Ribo B binding by mannosides reflects competition for ligand binding to the mannose receptor. In addition, it suggests that binding data from the bio-Ribo B assay are not significantly perturbed by heterogenicity of the sugar moiety from bio-Ribo B. The most complex mannosides from this study, M_5L_4 (**2d**) and M_6L_5 (**2e**) displayed nanomolar affinities for the mannose receptor, which are at least comparable to those of mannosylated albumin, [16] mannosylated poly-L-lysine [26,27] or endogenous glycoproteins such as t-PA. [9] Competition studies of ^{125}I-t-PA binding to endothelial liver cells confirmed the nanomolar affinity of M_6L_5. Previous efforts to synthesize low molecular weight ligands for the mannose receptor yielded compounds with 1000-fold lower, micromolar, affinities for the mannose receptor. [15,28,29] Oshimi *et al.* demonstrated that the affinity of tris(hydroxymethyl) based mannosides was only slightly higher than that of bi- and monosubstituted analogues, which was attributed to the suboptimal valency and spacing of the terminal mannosyl groups (≈9Å) within the mannose clusters. [28] The di-, tri- and tetramannosides synthesized by Robbins *et al.* [29] displayed higher, yet micromolar, affinities for the mannose receptor. Since the latter mannosides contained the same oligolysine backbone as our mannosides, their lower affinity is probably caused by differences in the chemical nature and the length of the spacers connecting the terminal α-D-mannose groups with the lysine backbone. In theory, the phenyl group of the (p-hydroxyanilino)carbamide spacer used in this study may contribute to ligand binding through π-π interaction forces. [30] Since preliminary binding studies revealed that the affinity of 4-aminophenyl-α-D-mannopyranoside is only slightly higher than that of α-D-mannose ($K_{i\,(app)}$= 1.8 mM, log (IC_{50}) = -2.48 ± 0.01, ; data not shown), we assume this

to be unlikely. An alternative explanation is that the (p-hydroxyanilino)carbamide spacer is longer (2Å) than the thioproprionyl spacer used by Robbins *et al.*[29] Recent studies by Biessen *et al.*[31] have already emphasized the relevance of optimal spacing of terminal glycosides to achieve avid recognition by a comparable eukaryotic lectin, the asialoglycoprotein receptor.

The $(\Delta G^{\circ})_{binding}$ from M_2L, calculated from the logarithm of the $K_{i\,(app)}$, is almost twice as large as that for α-D-mannose (-24.4 kJ/mol and -13.3 kJ/mol, resp.). In the case of competitive inhibition, this suggests that both mannose groups from M_2L participate similarly in the binding process. The binding energy for M_6L_5 (-45 kJ/mol) is, in turn, twofold higher than that of M_2L, suggesting that four α-D-mannose groups from M_6L_5 interact independently with the mannose receptor. This seems to contrast with the finding that the affinity increases steadily rather than stepwise with increasing mannose valency. This apparent paradox may be explained from the negative cooperative binding of M_3L_2-M_6L_5: binding of an additional α-D-mannose to the receptor may attenuate the association of already bound mannose groups. Alternatively, the observed gain in binding energy with mannose valencies ≥ 2 may arise from conformational effects. In that case, elongation of the cluster mannoside with a single mannose group may force adjacent mannose groups in a geometric configuration favourable for binding to the mannose receptor.

The complex nature of ligand binding by the mannose receptor was further illustrated by the Hill coefficients of the inhibition curves for cluster mannosides. The Hill coefficient of α-D-mannose was significantly larger than 1. From the nonlinear regression analysis according to a two-site mixed stimulation/inhibition model, it can be concluded that the high Hill coefficient reflects a compromise between a stimulatory effect of α-D-mannose at low concentrations (SC_{50}= 250 - 400 μM) and an inhibitory effect of α-D-mannose at an IC_{50} of 10 mM. Binding of the first α-D-mannose group to the high affinity site of the mannose receptor (possibly CRD4) may result in a 2-fold increased affinity of bio-t-PA or bio-Ribo B for the mannose receptor as was also reported by Oshimi et al.[28] In agreement with Oshimi *et al.*,[28] this stimulatory effect is less pronounced for moderately potent ligands like M_2L and absent for potent ligands like M_3L_2 to M_6L_5, as was reported in the same study.[28] At higher concentrations, bound ligand is displaced from the mannose receptor by binding of α-D-mannose to the low affinity site.

By contrast, M_3L_2 to M_6L_5 gave Hill coefficients of about 0.5 in both receptor sorbent assays and in the endothelial cell assay (M_6L_5), while Hill coefficients of bio-Ribo B or bio-t-PA indicated binding to a single site. Taking into account that the mannose receptor is a monomeric membrane-bound protein, this concurs with ligand binding to two (or more) separate but interacting sites within the mannose receptor. According to the binding model of Taylor and Drickamer *et al.*,[14] high affinity binding of proteins with high mannose-type glycosides like t-PA or ribonuclease B requires the cooperative action of CRD4 and CRD5. For optimal recognition of highly mannosylated polymers like mannan and invertase, the presence of five consecutive CRDs (CRD4-8) is imperative. Taken together it is tempting to identify CRD4 in concert with CRD5 as the first and CRD6-8 as the second binding unit. The first unit, embedding two carbohydrate recognition domains, is responsible for the binding of bio-Ribo B, bio-t-PA, α-D-mannose and M_2L. For binding of polyvalent mannosides like M_3L_2 to M_6L_5, and mannan, both the first (CRD4-5) and the second unit (CRD6-8) participate in the binding process. Nonetheless, further study will be needed to conclusively unravel the intriguing process of ligand binding to the mannose receptor.

In conclusion, we have devised and synthesized high affinity ligands for the mannose receptor, M_6L_5 being the most potent ligand yet synthesized. We anticipate that the nanomolar affinity of M_6L_5 is sufficiently high to allow application as a carrier device for cell-specific delivery of drugs/genes to macrophages and endothelial liver cells. Another relevant application which comes within reach involves antigen targeting to dendritic cells to stimulate major histocompatability complex-mediated antigen presentation by these cell type. Finally, M_6L_5 may also be a valuable therapeutic agent to prevent untimely clearance of the thrombolytic agent t-PA. In fact, preliminary studies in rats showed that administration of this mannoside resulted in a 2-fold delayed clearance of t-PA. [32]

ACKNOWLEDGEMENT

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CHAPTER 7

Cluster mannosides can inhibit mannose receptor-mediated tissue-type plasminogen activator degradation by both rat and human cells.

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SUMMARY

Recently we developed a series of cluster mannosides that were able to inhibit tissue-type plasminogen activator (t-PA) binding to the isolated mannose receptor. The mannoside with the highest affinity was able to inhibit t-PA clearance by the liver in the rat. To test whether these mannosides would also be efficient inhibitors in man, we studied the expression of the mannose receptor in the human liver and determined the efficacy of the mannosides to inhibit mannose receptor-mediated t-PA degradation by both rat and human cells.

Immunohistochemistry indicates that, like the rat, human liver endothelial cells and human Kupffer cells do express the mannose receptor. The mannosides do inhibit mannose receptor-mediated t-PA binding, association and degradation by isolated rat liver endothelial cells and t-PA association and degradation by cultured human macrophages at similar concentrations. The cluster mannoside with six mannose residues connected with a backbone of five lysine groups (M_6L_5) was, like unlabeled t-PA, able to inhibit ^{125}I-t-PA degradation in the nM range, while the mannoside M_5L_4 inhibited^{125} I-t-PA degradation in the μM range. The concentrations of mannoside necessary to inhibit ^{125}I-t-PA degradation *in vitro* were comparable to the concentrations necessary to inhibit mannose receptor-mediated ^{125}I-t-PA clearance *in vivo*.

We conclude that there is no species difference between rat and man with respect to the distribution of the mannose receptor in the liver and the affinity of the cluster mannosides, establishing the relevance of the inhibition of mannose receptor-mediated t-PA clearance by M_6L_5 as observed in the rat, for the human situation.

INTRODUCTION

Tissue-type plasminogen activator (t-PA) is a serine protease that activates fibrinolysis by converting plasminogen into plasmin, which cleaves fibrin into soluble degradation products. [1,2] Because of its fibrin-selective action, t-PA is successfully used for thrombolytic therapy, e.g., after myocardial infarction. [3] Recombinant t-PA (70 kD) contains a single high mannose-type oligosaccharide and one or two complex-type oligosaccharides. [4] In both rat and humans, t-PA is rapidly cleared mainly by the liver. [5,6] It has been shown that, in the rat, t-PA is cleared from plasma through both the mannose receptor and the α_2-macroglobulin receptor/ low density lipoprotein receptor-related protein (LRP). [5,7-10]

Because of its rapid clearance in man, large doses of t-PA must be administered to achieve efficient thrombolysis. Coadministration of inhibitors of t-PA clearance with t-PA would reduce the therapeutic dose of t-PA required for efficient thrombolysis. Furthermore myocardial infarction patients can have a large variability of liver blood flow.[11] The liver blood flow strongly influences the t-PA clearance. Reduced liver blood flow causes a decreased plasma clearance and increased plasma levels of endogenous or infused t-PA in humans. [12,13] Thus, reduced liver blood flow may lead to t-PA overdosing and an increased bleeding risk in patients. Coadministration of inhibitors of t-PA clearance would also reduce the influence of liver blood flow on the t-PA concentration, and thereby reduce the risk of t-PA overdosing. The inhibitors may in addition be used to inhibit the clearance of endogenous t-PA in order to enhance the fibrinolytic activity in blood and to prevent thrombosis.

We have shown that the uptake and degradation of t-PA by cultured human macrophages are mediated by both the mannose receptor and LRP, thus these cells are a good *in vitro* model for the plasma clearance of t-PA by the liver. [14] In the human liver the LRP is present on hepatocytes and Kupffer cells. [15] The mannose receptor has been shown to be present in rat [7,16] and bovine [17] liver on liver endothelial cells and Kupffer cells. The 175 kD mannose receptor can be isolated from human liver, [18] but its cellular localization in human liver has not yet been demonstrated.

Recently, we synthesized cluster mannosides with two to six terminal mannose residues connected to an (oligo)lysine backbone by flexible elongated spacers (M_2L_1 to M_6L_5). These mannosides can inhibit t-PA binding to the isolated immobilised mannose receptor. The affinity of the mannosides increased approximately 10 times from the µM to the (sub) nM range with each increment of a mannose residue attached to the backbone. [19] We showed that the most potent mannoside from this study (M_6L_5), in combination with an inhibitor of LRP, effectively inhibited t-PA plasma clearance in rats. [10] This indicated that the aforementioned strategy to delay t-PA clearance using mannose receptor inhibitors is feasible in the rat. No information is available about the effect of these mannosides on mannose receptor mediated t-PA degradation by human cells.

To explore the potentials of the cluster mannosides as mannose receptor antagonists or ligands in humans, we tested the mannosides in both rat and human models. We provided the first evidence for the presence of the mannose receptor on human liver endothelial cells and Kupffer cells by immunohistochemistry. Isolated rat liver endothelial cells and cultured human macrophages were used to test the efficacy of the cluster mannosides M_5L_4 and M_6L_5 to inhibit mannose receptor-mediated t-PA binding and/or degradation. The *in vitro* inhibitory effect of the mannosides was compared to the inhibitory effect of the mannosides on t-PA clearance *in vivo* in the rat.

MATERIALS AND METHODS

Materials

The synthesis and structure of the cluster mannosides M_6L_5, M_5L_4, M_4L_3, M_3L_2 and M_2L_1 was as described elsewhere. [19] Bovine serum albumin (BSA) fraction V and collagenase (type I and IV) were obtained from Sigma Chemical Co. (St.Louis MO, USA). Nycodenz was from Nycomed Pharma AS (Oslo, Norway) and 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid (HEPES) was from Merck (Darmstadt, FRG). Wistar rats were from the Sylvius Laboratories, (University of Leiden, The Netherlands). Human AB$^+$ serum, and thrombocyte-poor pooled buffy coats from healthy donor blood (pooled from 5 donors with the same blood type combined, and serum added to preserve the cells), were obtained from the Red Cross Blood Bank (The Hague and Leiden, The Netherlands). Lymphoprep™ (Nycomed Pharma AS, Oslo, Norway) with a density of 1.077 g/ml was used for density gradient centrifugation. Heparin (Leo Pharmaceutical Products, Ballerup, Denmark), cell culture medium M199 (Flow Laboratories, Irvine, UK), Penicillin/Streptomycin (Pen/strep, Boehringer Mannheim, Mannheim, Germany), and sterile buffers were used to isolate and culture the monocytes. Dexamethasone was purchased from Sigma Chemical Co. (St.Louis, MO, USA). Recombinant t-PA (Actilyse) was obtained from Boehringer Ingelheim (Ingelheim, Germany). Mannan extracted from *Saccharomyces cerevisiae*, prepared by the cetavlon method was obtained from Sigma Chemical Co. (St.Louis MO, USA). The fusion protein of glutathione-S-transferase and α_2-macroglobulin receptor-associated protein (GST-RAP) was a generous gift from Dr J. Kuiper who used the *Salmonella japonicum* glutathione-S-transferase (GST)-RAP expression plasmid. [20] (provided by Dr. J.Herz, University of Texas, Southwestern Medical Center, Dallas) for synthesis. Mouse monoclonal antibody KP-1 (isotype IgG_1) generated against the CD68 antigen was obtained from Dako A/S (Glostrup, Denmark). Mouse monoclonal antibody 15-2 (isotype IgG_1) generated against the isolated human mannose receptor was prepared as described previously. [21] Human liver cryostat sections were obtained from biopsy samples and snap-frozen in liquid nitrogen (Department of Pathology, Slotervaart Hospital, Amsterdam, The Netherlands).

Immunohistochemistry of the mannose receptor

Tissue sections of 8 µm thickness were cut on a Reichert-Jung 2800 frigocut cryostat, transferred to poly-L-lysine-coated microscope slides, air dried for 30 min at room temperature and fixed in acetone/chloroform 1:1 (10 min at 4°C). After washing with phosphate buffered saline (PBS) the tissue sections were pre-incubated with normal rabbit serum for 5 minutes. Excess serum was

discarded and the sections were incubated with primary antibody (mAb 15-2 or KP-1) for 30 min at room temperature. After rinsing in PBS, the sections were incubated with secondary antibody (rabbit anti-mouse, Dako A/S, Glostrup, Denmark) for 15 min at room temperature. After rinsing with PBS the sections were incubated for 30 min with an alkaline-phosphatase-conjugated mouse monoclonal antibody directed against alkaline phosphatase (Dako A/S, Glostrup, Denmark). The alkaline phosphatase was visualized in red by incubating the section for 30 min in the dark with naphtol-AS-MX-phosphate (Sigma Chemical Co., St.Louis, MO) and New Fuchsine (Chroma Gezellschaft, Köngen, BRD) as a coupling salt. [21,22] The alkaline phosphatase was visualized in blue by incubating the section for 30 min at room temperature in the dark with a modified substrate solution described by Cordell et al.. [23] The solution was prepared by dissolving naphtol-AS-MX-phosphate in dimethylformamide and 0.2 M Tris-HCl pH 8.0. Directly before use 0.5 mg/ml Fast Blue BB and 0.05 mg/ml levamisole (Sigma Chemical Co. St.Louis, MO) were added, and the solution was filtered.

To obtain a double staining for both the mannose receptor and CD68, the above-mentioned procedure was performed with KP-1 as the first primary antibody and the alkaline phosphatase was stained red for 30 min. The stained sections were extensively washed with PBS and incubated for 5 min with normal rabbit serum, and the above-mentioned procedure was repeated now with mAb 15-2 as the primary antibody, and the alkaline phosphatase was stained blue for 15 min.

Finally the sections were rinsed with *aqua dest*, counterstained with either Mayers haematoxylin (blue) or Fast nuclear red (red), air dried, and mounted in DePeX mounting medium (BDH, Poole, UK). In control sections no primary antibody was used. The identity of the cells was judged on the basis of their morphology and location in the tissue.

Rat liver endothelial cell isolation

In this study male Whistar rats were used which had free access to standard chow diet (Hope farms, Woerden, The Netherlands) and tap water; they were kept on a 12 hr day/night cycle and received humane care. Rats (12-weeks old males) were anaesthesized by intraperitoneal injection with 20 mg of pentobarbital. The vena porta was cannulated and the liver was perfused with Hank's buffer supplemented with Hepes and collagenase for 20 minutes at 37°C. The liver endothelial cells were isolated by use of density gradient centrifugation (Nycodenz) and centrifugal elutriation. [24] Purity (>99%) and viability (>90%) were judged by respectively peroxidase staining (Kupffer cell staining) and trypan blue exclusion.

Monocyte isolation and cultivation

Monocytes were isolated from pooled human buffy coats as described earlier. [14] Buffy coat (100 ml) was diluted with 180 ml phosphate buffered saline (PBS, 2.7 mM KCl, 137 mM NaCl, 8.1 mM Na_2HPO_4, 1.46 mM KH_2PO_4, pH 7.4) containing 10 units/ml heparin. Using density gradient centrifugation with Lymphoprep, routinely 5 to 10 $x10^8$ cells were obtained. These cells were resuspended in culture medium (M199 containing 10 mg/ml glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin) and 5 x 10^6 cells/ml were seeded in 24-well culture plates. After one hour, the nonadherent cells were removed with medium, and cells were cultured in culture medium containing 0.1 µg/ml dexamethasone and 10% (v/v) human AB^+ serum. After 2 days the monocytes were differentiated to macrophages and used for degradation studies.

Labeling of t-PA

Recombinant t-PA was labeled with ^{125}I using the iodogen method. [14] Polyethylene vessels of 1.5 ml were coated with 10 µg iodogen. 50 µg t-PA in 0.2 M arginine pH 8, was incubated with 0.5 mCi $Na^{125}I$ for 10 min at room temperature in the vessel. The mixture was transferred to another vessel containing an equal volume of 4 mg/ml KI to avoid non-specific binding of ^{125}I to t-PA. Labeled t-PA was separated from free ^{125}I using a 10 ml Sephadex G25 Coarse column (eluent 50 mM Tris-HCl, 1 M NaCl, 0.01% (v/v) Tween 80, 1 mg/ml BSA, pH 8). Labeling resulted in approximately 600 cpm/fmol, with 80% recovery of t-PA activity.

Binding, association and degradation of ^{125}I-t-PA by rat liver endothelial cells

As described previously, [7] freshly isolated rat liver endothelial cells were incubated with 1 nM ^{125}I-t-PA in HAM's F10 buffer supplemented with 25 mM Hepes, 20 mg/ml BSA, and 0.01% (v/v) Tween 80, pH 7.4 at 4°C (binding 2 hr) or 37°C (association 15 min and degradation 1 hr) under constant rotation. After incubation the cells were washed twice with cold 50 mM Tris-HCL, 0.15 M NaCl, 2.5 mM $CaCl_2$, 2 mg/ml BSA, 0.01% Tween 80, pH 7.4, and once with this buffer without BSA. The cells were resuspended in water and radioactivity was measured. Degradation products in the medium were measured after precipitation with 10% (w/v) trichloroacetic acid (TCA) and subsequent extraction of the supernatant with chloroform (see below). Binding, association and degradation were measured and corrected for protein, as measured according to the Lowry method. Specific binding, association or degradation were defined as the residual binding, association and degradation after subtraction of radioactivity determined in the presence of 1 mg/ml mannan or 100 mM mannose. Under the current conditions the non-specific binding was 14.3 ± 6.4 % , the non-specific association was 3.7 ± 3.8 % and the non-specific degradation was 8.3 ± 5.2 % of control).

Association and degradation of ^{125}I-t-PA by human macrophages

As described previously. [14] the macrophages were washed (3x with PBS containing 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mg/ml BSA,

pH 7.4, 4°C), and incubated with 1 to 17 nM ^{125}I-t-PA and 100 nM GST-RAP (for association experiments 20 mM 6-aminohexanoic acid was also added), with or without mannosides in 300 µl M199, 10 mg/ml BSA, 0.01% (v/v) Tween 80, at 37°C in 5% CO_2/ 95% air. After incubation, cell media were collected and the cells were washed four times with PBS, pH 7.4 containing 20 mM 6-aminohexanoic acid and 0.1% BSA and lysed with 500 µl 1% Triton X-100 for 15 minutes at room temperature. The lysates were collected and another 500 µl 1% Triton X-100 was added to the wells, collected and added to the above-mentioned lysate. Radioactivity of the lysates was determined. Specific association was defined as the residual binding after subtraction of radioactivity determined in the presence of 1 mg/ml mannan. Under the current conditions the non-specific association was 19.8 ± 8.6% of control. TCA was added to the cell media (final conc. 10% w/v). Non-degraded ^{125}I-t-PA was precipitated by centrifugation (10 min 15000 g). To eliminate the possibility that the cells might deiodinate rather than degrade ^{125}I-t-PA, free ^{125}I was extracted. To 500 µl TCA soluble supernatant obtained, 5 µl 400 mg/ml KI and 25 µl H_2O_2 30% (v/v) were added. After 5 minutes the free iodine was extracted with 800 µl chloroform. The radioactivity of the remaining ^{125}I-tyrosine (and possibly ^{125}I-peptides) in the upper layer (5 min 15000 g) representing degraded ^{125}I-t-PA was determined. Degradation was corrected for radioactivity determined in wells incubated without cells. Specific degradation was defined as the residual degradation after subtraction of radioactivity determined in the presence of 1 mg/ml mannan. Under the current conditions the non-specific degradation was 6.0 ± 4.8 % of control.

Analysis of inhibition curves

From the inhibition data of the binding, association or degradation of non-saturating concentrations of ^{125}I-t-PA by unlabeled t-PA or other compounds, specific ^{125}I-t-PA binding, association and degradation were calculated and expressed as a percentage of control. To calculate the binding parameters of the inhibitors (log half maximal inhibitory concentration (log IC_{50}), and Hill-slopes) the resulting inhibition curves were fitted as a sigmoid curve using nonlinear regression analysis with the computer program GraphPAD (ISI Software, Philadelphia, PA, USA). "Goodness of fit" was assessed by evaluating the actual distance of the measurements from the fitted line (no weighing).

Plasma clearance of therapeutic doses of t-PA in the rat

Twelve-week-old male Wistar rats (225-275 g) were anesthetized by intraperitoneal injection with 20 mg of pentobarbital. The abdomen was opened. Inhibitor in 250 µl PBS was injected into the vena cava one minute before 600 µg/kg radiolabeled t-PA in 500 µl PBS was injected. At 1, 2, 5, 10, 20, and 30 min blood samples (0.3 ml) were taken with heparinized syringes from the vena cava. Concentration of t-PA in plasma was calculated as the percentage of the injected dose, and clearance was expressed as the area under the curve (AUC) from 0 to 30 minutes.

RESULTS

Recently we developed a mouse monoclonal antibody (mAb 15-2) against the human mannose receptor. By the use of mAb 15-2, we can specifically detect mannose receptor-expressing human macrophages in flow cytometry and in cryostat sections of human tissue.[22] mAb KP-1 detects the CD68 antigen expressed by myeloid cells.[25] It has the same isotype as mAb 15-2, and thus also served as a negative control. We compared the staining patterns of these two mAb in single and double staining of cryostat sections of human liver. As shown in Fig 1 (see appendix, page 148,149) the CD68 antigen was only present on Kupffer cells whereas the mannose receptor was present on both human liver endothelial cells and Kupffer cells. No staining was observed in controls.

To evaluate the affinity of the cluster mannosides for the mannose receptor as expressed on cells, we tested their ability to inhibit t-PA binding to rat liver endothelial cells at 4°C (Fig 2). Previously, we have shown that M_6L_5 inhibited t-PA binding to rat liver endothelial cells in the nM range. Here we show that M_5L_4 inhibited t-PA binding in the µM range. The mannosides M_4L_3, M_3L_2, M_2L_1 all inhibited t-PA binding like M_5L_4 in the µM range (IC_{50} 4-7 µM), while mannose inhibited t-PA binding in the mM range (IC_{50} 3 mM) (not shown). At 37°C, t-PA bound to the mannose receptor is internalized, the ligand is released in the endosomes and the mannose receptor is recycled to the membrane. After a lag phase of 10 to 15 minutes degradation products of t-PA appear in the medium.[7,14] We evaluated the M_6L_5 and M_5L_4 inhibition of t-PA binding and internalization (association) for 15 min at 37°C by rat liver endo-thelial cells (Fig 3). The association was inhibited at similar mannoside concentrations as the binding at 4°C. We therefore conclude that the temperature did not influence the efficacy of the inhibitors.

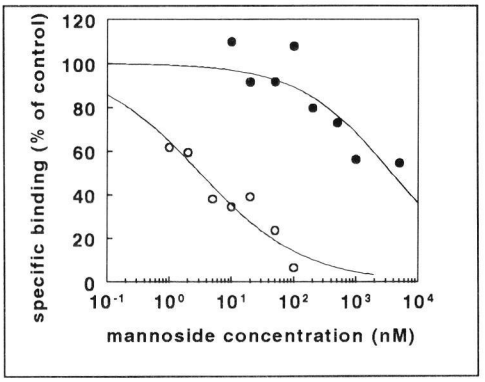


Fig 2 M_6L_5 and M_5L_4 inhibition of ^{125}I-t-PA binding to isolated rat liver endothelial cells at 4°C.
Rat liver endothelial cells were incubated with ^{125}I-t-PA and different concentrations of inhibitor for 2 hours at 4 °C. The cells were washed, and bound radioactivity was determined. Data were corrected for non-mannose receptor specific binding and expressed as a percentage of control. M_6L_5 (○), n=2, M_5L_4 (●), n=2.

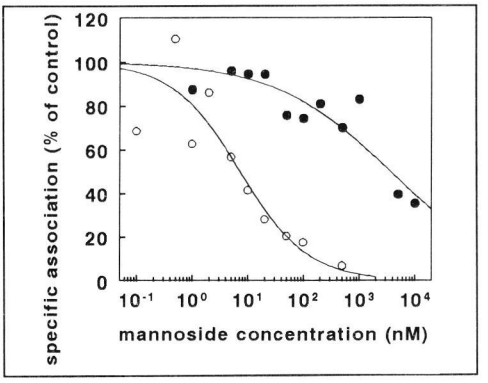


Fig 3 M_6L_5 and M_5L_4 inhibition of ^{125}I-t-PA association to isolated liver endothelial cells at 37°C.
Rat liver endothelial cells were incubated with ^{125}I-t-PA and different concentrations of inhibitor for 15 minutes at 37 °C. The cells were washed and bound radioactivity was determined. Data were corrected for non-mannose receptor specific association and expressed as a percentage of control. M_6L_5 (○) n=1, M_5L_4 (●), n=2.

To study the effect of the mannosides on human cells we cultured isolated human monocytes that differentiated to macrophages. As human macrophages express both the mannose receptor and LRP they are a suitable *in vitro* model to study t-PA clearance. [14] To enhance the ratio of mannose receptor mediated t-PA degradation we up-regulated mannose receptor expression using dexamethasone. [14] At 4°C, only a small amount of t-PA binds to the macrophages. Of this binding, approximately 50% is inhibited by the lysine analog 6-aminohexanoic acid (that does not affect t-PA degradation [14]), whereas only 10% of the binding is inhibited by mannan and 10% by GST-RAP. Thus the macrophages were not suitable for studying mannose receptor-mediated binding of t-PA at 4 °C. Like the mannose receptor on rat liver endothelial cells the mannose receptor on macrophages is recycled at 37 °C, and t-PA mannose receptor-mediated association was studied for 1 hour in human macrophages in the presence of GST-RAP and 6-aminohexanoic acid. As shown in Fig 4, M_6L_5 and M_5L_4 inhibited mannose receptor-mediated t-PA association by human macrophages in the nM range and the µM range, respectively.

To study the mannose receptor-mediated t-PA degradation, LRP-mediated t-PA degradation was inhibited using GST-RAP. [14] Under these conditions t-PA degradation is inhibited by 1000 nM unlabeled t-PA for 95% (Fig 5) and by 1 mg/ml mannan for 94% (not shown). As shown in Fig 6 the degradation of t-PA was inhibited by M_6L_5 with a similar affinity as unlabeled t-PA in both the presence and the absence of GST-RAP. Because the inhibition in the absence and presence of GST-RAP was similar, we may conclude that LRP-mediated t-PA degradation was not affected by M_6L_5. Furthermore the compound did not appear to be toxic to the human macrophages, because no cell death was observed and the inhibitory activity of 5 µM M_6L_5 was maintained at a constant level (81.3 ± 5.2 %) for at least 6 hours (Fig 7).

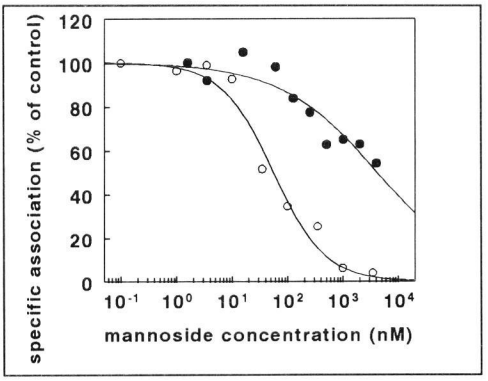


Fig 4 M_6L_5 and M_5L_4 inhibition of ^{125}I-t-PA association to cultured human macrophages at 37°C.

Human macrophages were incubated with ^{125}I-t-PA in the presence of 100 nM GST-RAP, 20 mM 6-amino-hexanoic acid and different concentrations of mannosides. The cells were incubated for 1 hour at 37 °C. Cells were washed and lysed, and ^{125}I-t-PA association was determined. Data were corrected for non-mannose receptor specific association and expressed as a percentage of control. M_6L_5 (○), n=2, M_5L_4 (●), n=2.

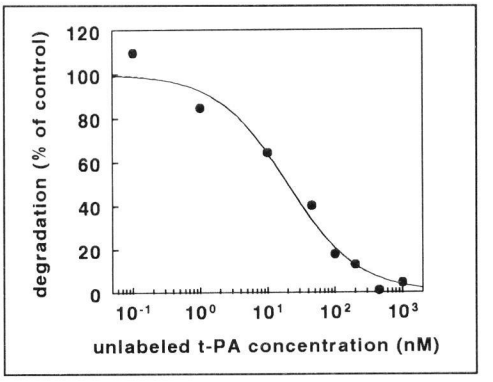


Fig 5 t-PA inhibition of ^{125}I-t-PA degradation by cultured human macrophages in the presence of GST-RAP.

Human macrophages were incubated with ^{125}I-t-PA in the presence of 100 nM GST-RAP and different concentrations of unlabeled t-PA for 1 hour at 37 °C. Medium was sampled and degradation of ^{125}I-t-PA was determined. Data were expressed as a percentage of control.

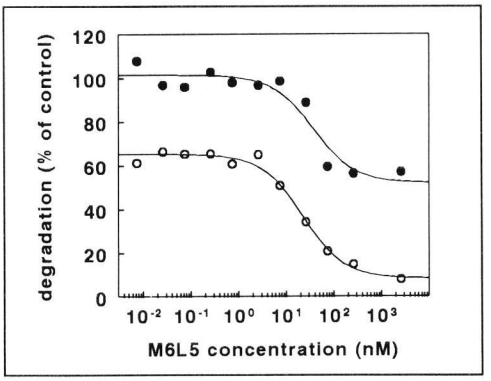


Fig 6 M_6L_5 inhibition of ^{125}I-t-PA degradation by human macrophages in the presence or absence of GST-RAP.

Human macrophages were incubated with ^{125}I-t-PA and different concentrations of inhibitor for 1 hour at 37 °C. Medium was sampled and the degradation product of ^{125}I-t-PA was determined. Data were expressed as a percentage of control. Inhibition in the presence of 100 nM GST-RAP (○), n=2, inhibition in the absence of GST-RAP (●), n=2.

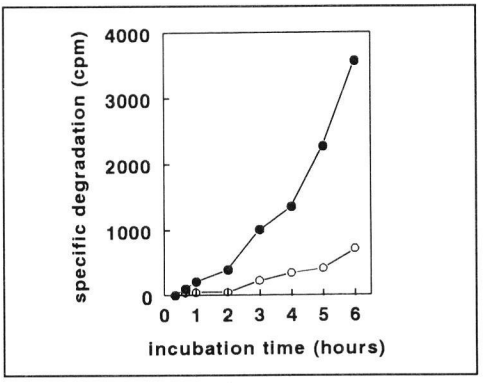


Fig 7 M_6L_5 inhibition of ^{125}I-t-PA degradation by human macrophages at various times of incubation.

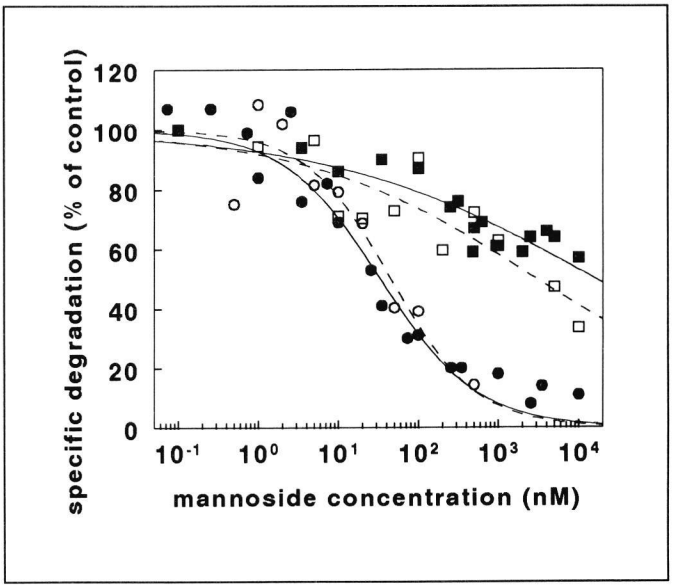
Human macrophages were incubated with ^{125}I-t-PA and 100 nM GST-RAP with or without 5 μM M_6L_5 for various periods in separate wells. From each well, medium was sampled, and the degradation of t-PA was determined. Data were corrected for non-mannose receptor specific degradation and expressed as the specific degradation cpm present in the sample. Control degradation (●), n=2), degradation in the presence of 5 μM M_6L_5 (○), n=2.

Subsequently, the mannoside inhibition of mannose receptor-mediated t-PA degradation was determined in both human macrophages and rat liver endothelial cells over an incubation period of 1 hour at 37°C (Fig 8). The mannose receptor-mediated t-PA degradation by both cell types was inhibited at similar concentrations of M_6L_5 (IC_{50} 30-50 nM) and M_5L_4 (IC_{50} 3-16 µM). We conclude that there is no species difference between rat and man with respect to the affinities of M_6L_5 and M_5L_4 toward the mannose receptor.

Fig 8

M_6L_5 and M_5L_4 inhibition of ^{125}I-t-PA degradation by isolated rat liver endothelial cells and cultured human macrophages.

Human macrophages were incubated with ^{125}I-t-PA and 100 nM GST-RAP, while rat liver endothelial cells were incubated with ^{125}I-t-PA. Both cell-types were coincubated with different concentrations of inhibitor for 1 hour at 37 °C. Medium was sampled, and the degradation of ^{125}-t-PA was determined. Data were corrected for non-mannose receptor specific degradation and expressed as a percentage of control. Inhibition curves obtained with rat liver endothelial cells are represented by a dotted line, those obtained with human macrophages by a solid line. M_6L_5 and rat cells (○) n=2, M_6L_5 and human cells (●) n=6, M_5L_4 and rat cells (□), n=2, M_5L_4 and human cells (■), n=6.



To evaluate the predictive value of the *in vitro* assays for *in vivo* inhibition of mannose receptor-mediated t-PA clearance, we tested the ability of cluster mannoside M_5L_4 to inhibit t-PA clearance at 0.3 mg/rat and compared these results to the recently described effect of two different doses of M_6L_5.[10] As shown in Table 1, M_5L_4 inhibited t-PA clearance for only 38.9% at a dose of 0.3 mg, which is significantly lower than the 58.7% inhibition achieved with the same dose of M_6L_5. Even a ten times lower dose of M_6L_5 (0.03 mg) gave a stronger inhibition of t-PA clearance (47.6%) than M_5L_4. Comparison of the potency of the mannosides to inhibit t-PA degradation *in vitro* with the inhibition of t-PA clearance *in vivo* (see discussion) suggests that it is possible to extrapolate the effects observed on t-PA degradation by isolated cells (rat and human) to the effects observed on t-PA clearance in the rat.

DISCUSSION

In both rat and man the main clearance organ of t-PA is the liver.[5,6] Recently we developed a monoclonal antibody (mAb 15-2) that specifically recognizes the 175 kD human mannose receptor and not other mannose binding proteins or mannose receptor-related proteins [21,22] By the use of mAb 15-2 we show for the first time that there is no species difference concerning the distribution of the mannose receptor in the liver because it is present on human liver endothelial and Kupffer cells similar to the bovine liver and rat liver.[7,16,17]

TABLE 1 **Inhibition of t-PA clearance in the rat by intravenous administration of the cluster mannosides.**

inhibitor	inhibitor concentration (μM)*	AUC of t-PA (%dose.min)	inhibition of t-PA clearance (%)
control	0	231 ± 45	0
M_5L_4 (0.3 mg)	2.8 - 17	379 ± 20	38.9
M_6L_5 (0.03 mg)	0.24 - 1.5	442 ± 59	47.6
M_6L_5 (0.3 mg)	2.4 - 15	560 ± 47	58.7

*Calculated range of the plasma concentration of the inhibitor after injection when the inhibitor is distributed over total body fluid (approximately 50 ml) or only the plasma volume (approximately 8 ml)

Recently we developed cluster mannosides which were able to inhibit binding of t-PA to the isolated mannose receptor [19] and t-PA clearance in the rat. [10] To explore possible applications of the cluster mannosides as mannose receptor antagonists or ligands in man, for example to improve the pharmacokinetic properties of t-PA, we compared their effect on t-PA degradation by mannose receptor expressing rat cells with their effect on human cells *in vitro*.

The mannosides M_6L_5 and M_5L_4 inhibited mannose receptor-mediated t-PA binding and association by rat liver endothelial cells. M_6L_5 did not affect LRP-mediated t-PA degradation by human macrophages, nor did it show toxicity towards these cells. This is in agreement with previous findings showing that no toxicity of 0.3 mg M_6L_5 could be detected *in vivo* in the rat. [10] Furthermore we showed that the mannose receptor-mediated t-PA association and degradation by rat liver endothelial cells and human macrophages was similarly inhibited by the mannosides. On both celltypes, M_6L_5 inhibited t-PA degradation in the nM range (IC_{50} 30-50 nM) and M_5L_4 in the μM range (IC_{50} 3 - 16 μM). We therefore may conclude that the mannosides do not display any species difference or cell-type difference in their affinities towards the mannose receptor.

The large difference in affinity of the two mannosides M_6L_5 and M_5L_4 is in line with the results obtained by other groups. Synthetic lysine-based cluster mannosides with two to four mannose residues had affinities in the mM to μM range towards isolated macrophages, [26,27] whereas inhibitors with polymannose structures like $mannose_{48}$-BSA and $mannose_{104}$-polylysine had affinities for macrophages in the nM range. [28,29] Using isolated hybrid and high-mannose type oligosaccharides, it was found that oligosaccharides with tri-antennary mannose residues are endocytosed by rat liver endothelial cells with an affinity of 60-600 nM, whereas oligosaccharides with di-antennary mannose residues are not detectably endocytosed. [30] The mannose receptor is involved in the uptake of antigen by monocyte-derived dendritic cells. [31] It was reported that high-mannose type oligosaccharides with di- or tri-antennary mannose residues inhibit antigen uptake in the μM range, whereas longer tri-antennary oligosaccharides inhibit antigen uptake in the nM range. [32] These data all indicate that, for binding to the mannose receptor with nM affinity, the ligand must contain multiple mannose residues located at some distance from each other.

The mannose receptor contains 8 carbohydrate recognition domains (CRD). [33] It was found that the receptor has a high affinity for mannan (Ki 110 nM). [34] When truncated receptor forms were expressed it was concluded that CRD 4,5 and 7 are essential for the high affinity binding (nM range) of mannan, because mannan has a much lower affinity (μM to mM range) for mutant receptors lacking one of these CRDs. [34,35] It was also found that CRD 4 is essential for efficient endocytosis because receptor variants with CRD 5-8 have a much lower endocytosis rate than receptors with CRD 4-8. [34]

The above-mentioned differences in affinity for the mannose receptor of mannosides with a different chain length and the differences of the mannan affinity for various truncated mannose receptors indicates that the chain length of the mannoside determines the number of CRDs it can bind to, and, thereby, the affinity of the ligand for the mannose receptor. Our results suggested that t-PA and M_6L_5 had the proper configuration to bind with high affinity to the mannose receptor expressed on cells. Because the CRD 4,5 and 7 are essential for nM binding affinity to the receptor, we hypothesize that one molecule of t-PA or M_6L_5 binds simultaneously to the mannose receptor CRD 4,5 and 7, which results in high affinity binding. It is possible that the cluster mannoside M_5L_4 is too short and one molecule of mannoside may only bind to two out of three CRDs which results in a much lower binding affinity.

We compared our *in vitro* data with *in vivo* data. In rats, the clearance of mutant t-PA (point mutation) which lacks the oligomannose residue is about 57% compared to normal glycosylated t-PA. [36] This is similar to the observed inhibition of t-PA clearance with the highest dose of M_6L_5 we used. When the inhibition by 0.3 mg M_6L_5 is considered to be maximal (100%), the observed inhibition of mannose receptor mediated clearance with 0.3 mg M_5L_4 is 66%, and, with 0.03 mg M_6L_5 , it is 81%. When we assume that the mannosides are maximally distributed over the total body fluid (approximately 50 ml) and minimally only in plasma (approximately 8 ml) the mannosides reach plasma concentrations as shown in table 1. Within this estimated plasma concentration range of the mannosides the degradation of t-PA by cells would have been inhibited with M_5L_4 by 44% to 56%, with M_6L_5 (dose 0.03 mg) by 80% to 91%, and with M_6L_5 (dose 0.3 mg) by 96% to 99%. This analysis suggests that the mannose receptor functioning *in vitro* on isolated rat liver endothelial cells and human macrophages is comparable to and representative of the functioning of the mannose receptor *in vivo* in the rat.

The mannose receptor also plays a role in the immune system in antigen uptake by antigen-presenting cells. [31] and in the uptake of intracellular parasites such as *Leishmania donovani*,[37] *Tryponosoma cruzi*. [38] and *Mycobacterium tuberculosis*. [39] As M_6L_5 has a high affinity for the mannose receptor and inhibits t-PA binding, it may also be used to inhibit the uptake of antigens or pathogens. Furthermore M_6L_5 could be a good candidate for drug targeting towards mannose receptor-expressing cells. [40,41] Further studies are required to substantiate these potential therapeutic applications of the high affinity mannose receptor ligand M_6L_5.

In conclusion, we demonstrate that, as in the bovine and rat liver, the mannose receptor is present on liver endothelial cells and Kupffer cells in human liver. The newly synthesized cluster mannoside M_6L_5 inhibits mannose receptor t-PA binding, uptake and degradation with high affinity (unlike smaller cluster mannosides) on both rat and human cells. t-PA is cleared mainly by the liver in both species. M_6L_5 is a potent inhibitor of mannose receptor-mediated t-PA clearance in the rat, and we expect that it is capable of inhibiting the mannose receptor-mediated t-PA clearance in man to a similar extent.

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CHAPTER 8

Inhibition of mannose receptor-mediated clearance of tissue-type plasminogen activator (t-PA) by dextran: a new explanation for its antithrombotic effect.

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SUMMARY

Dextran is used during surgery as a prophylactic agent to prevent deep venous thrombosis. Recently it has been shown that dextran increases t-PA plasma concentrations in patients. As dextran is a potential ligand for the mannose receptor, we studied whether this glucose-polymer would be able to inhibit mannose receptor-mediated clearance of t-PA.

In this report we show that dextran 40 and dextran 70 were able to inhibit t-PA binding to the isolated human mannose receptor (IC_{50} 14 and 4 mg/ml, respectively). Both glucose-polymers inhibited mannose receptor-mediated t-PA degradation by human monocyte-derived macrophages *in vitro* (IC_{50} 7 and 2 mg/ml, respectively). The α_2-macroglobulin receptor/ low density lipoprotein receptor-related protein (LRP)-mediated t-PA degradation by the macrophages was not affected by dextran. During and after a 45-min infusion of dextran 70 (Macrodex) in rats, in plasma endogenous t-PA concentrations increased to 162 ± 33% and 122 ± 35% respectively. The plasma clearance of a bolus injection of exogenous t-PA was decreased by 33 ± 9% in the same rats.

We conclude that dextran inhibits mannose receptor-mediated t-PA clearance. The inhibition of t-PA clearance during dextran infusion results in increased endogenous t-PA plasma concentrations. Increased t-PA concentrations present during thrombus formation are known to increase thrombus lysability. Thus the inhibition of t-PA clearance can contribute to the antithrombotic effect of dextran.

INTRODUCTION

Tissue-type plasminogen activator (t-PA) is a serine protease that activates fibrinolysis by converting plasminogen into plasmin, which cleaves fibrin into soluble degradation products. [1,2] Because of its fibrin selective action, t-PA is successfully used for thrombolytic therapy, for instance after myocardial infarction. [3] Recombinant t-PA is a glycoprotein which contains a single high mannose-type and one or two complex-type oligosaccharides. [4] It is rapidly cleared from plasma by the mannose receptor and the α_2-macroglobulin receptor/ low density lipoprotein receptor-related protein (LRP). [5-7]

Previously we found that synthesized cluster mannosides are able to inhibit the binding of t-PA to the isolated mannose receptor, [8] to inhibit mannose receptor-mediated degradation by rat and human cells (Noorman et al. Hepatology 1997, in press), and to inhibit the clearance of t-PA in the rat. [7] We wanted to know whether an inhibitor of mannose receptor-mediated t-PA degradation would increase the plasma concentrations of endogenous t-PA in man and thereby prevent thrombosis in man. For this we searched the literature for potential ligands/inhibitors of the mannose receptor which have been shown to increase plasma t-PA concentrations in man.

Dextran is a potential ligand for the mannose receptor expressed on monocyte-derived immature dendritic cells. [9] It is a neutral polysaccharide, which is synthesized from saccharose by a number of bacterial species of the family of the Lactobacillae. It consists of glucose units linked together by alpha 1-6 bonds, and at intervals of about 20 glucose units, short side branches are attached to the main chain by alpha 1-3 linkages. [10] By partial hydrolysis dextran is made with mean molecular weights of 40,000 (Rheomacrodex or dextran 40) or 70,000 (Macrodex or dextran 70). These dextrans are clinically used as plasma volume expanders for the efficient resuscitation from haemorrhagic hypotension and as a blood substitute in surgery. [11,12] It is found that dextran is also effective as a prophylactic agent during

surgery to prevent deep venous thrombosis. [13-16] Recently it has been shown that t-PA antigen in plasma was increased during dextran infusions in surgical patients by an unknown mechanism. [17]

Since dextran is a potential ligand of the mannose receptor, we hypothesized that the increased t-PA concentrations might have been caused by inhibition of mannose receptor-mediated t-PA clearance. In the present study we tested whether t-PA binding to the isolated human mannose receptor is inhibited by therapeutic concentrations of dextran. We also tested the effect of dextran on t-PA degradation mediated by the mannose receptor and LRP expressed on human macrophages, an *in vitro* model for the plasma t-PA clearance by the liver. [18] Finally we compared the effect of 6% dextran 70 in 0.9% NaCl infusion with the effect of 0.9% NaCl infusion on the plasma concentration of endogenous t-PA and on the clearance of a bolus injection of exogenous t-PA *in vivo* in the rat.

MATERIALS AND METHODS

Materials

N-hydroxysuccinimide-biotin (NHS-biotin, Zymed Laboratories Inc., South San Francisco, CA, USA), highly activated polyvinyl chloride microtitre plates (Micronic b.v., Lelystad, The Netherlands), alkaline phosphatase-conjugated streptavidine (Amersham, Buckinghamshire, UK), and p-nitrophenyl phosphate (Sigma, St. Louis, MO, USA) were used for the immobilised mannose receptor assay. Human AB$^+$ serum, and thrombocyte-poor pooled buffy coats from healthy donor blood (pooled from 5 donors with the same blood type combined, and serum added to preserve the cells), were obtained from the Red Cross Blood Bank (The Hague and Leiden, The Netherlands). Lymphoprep™ (Nycomed Pharma AS, Oslo, Norway) with a density of 1.077 g/ml was used for density gradient centrifugation. Heparin (Leo Pharmaceutical Products, Ballerup, Denmark), cell culture medium M199 (Flow Laboratories, Irvine, UK), Penicillin/Streptomycin (Pen/strep, Boehringer Mannheim, Mannheim, Germany), and sterile buffers were used to isolate and culture the monocytes. Dexamethasone was purchased from Sigma Chemical Co. (St.Louis, MO, USA). Tissue-type plasminogen activator (t-PA), was purified from a recombinant human melanoma cell culture [19] by Dr. J.H. Verheijen of our laboratory. Recombinant t-PA (Actilyse) was obtained from Boehringer Ingelheim (Ingelheim, Germany). Bovine serum albumin (BSA) fraction V and mannan extracted from *Saccharomyces cerevisiae*, prepared by the cetavlon method were obtained from the Sigma Chemical Co. (St.Louis, MO, USA). The fusion protein of glutathione-S-transferase and α_2-macroglobulin receptor-associated protein (GST-RAP) was prepared by Dr. J. Kuiper (Sylvius Laboratory, Leiden) using the *Salmonella japonicum* glutathione-S-transferase (GST)-RAP expression plasmid [20] (kindly provided by Dr. J.Herz, University of Texas, Southwestern Medical Center, Dallas). Dextran T40 and Dextran T70 and Macrodex (6% Dextran 70 in 0.9% NaCl) were obtained from Pharmacia Fine Chemicals AB (Uppsala, Sweden).

Inhibition of t-PA binding to the immobilised mannose receptor.

Melanoma t-PA (0.1 mg/ml) was dialysed against 0.1 M $NaHCO_3$, 0.01% (v/v) Tween 80, pH 8.4 and incubated with NHS-biotin at room temperature for 3 hours at molar ratio 1:200. After incubation biotinylated-t-PA (biot-t-PA) was dialysed against 20 mM Tris/HCl, 150 mM NaCl, 0.01% (v/v) Tween 80, pH 7.4. Mannose receptor was isolated and purified from human placenta on a mannosylated-albumin-Sepharose column. [21,22] The receptor was adsorbed overnight at 4 C to microtitreplates in 20 mM Tris/HCl buffer, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.4. The wells were incubated for 0.5 h at room temperature with binding buffer (20 mM Tris/HCl, pH 7.4 containing 0.5% (v/v) Tween 80, 150 mM NaCl, 5 mM $CaCl_2$, and 1 mg/ml BSA). The immobilised receptor was incubated with varying concentrations of inhibitor in binding buffer for 0.5 h. Subsequently a nonsaturating concentration of biot-t-PA (100 ng/ml, final concentration) was added and the mixture was incubated at room temperature for 2 h. Bound biot-t-PA was quantified by incubation at room temperature for 1 h with alkaline phosphatase-conjugated streptavidine. Between each incubation step the wells were washed 3 times with binding buffer. Bound conjugate was detected by measuring the conversion rate of the chromogenic substrate p-nitrophenyl phosphate at 25°C. The results were corrected for non-specific binding obtained in wells which were "coated" with buffer without mannose receptor.

Monocyte isolation and cultivation.

Monocytes were isolated from pooled human buffy coats as described earlier. [18] Buffy coat (100 ml) was diluted with 180 ml phosphate buffered saline (2.7 mM KCl, 137 mM NaCl, 8.1 mM Na_2HPO_4, 1.46 mM KH_2PO_4, pH 7.4) containing 10 units/ml heparin. Using density gradient centrifugation with Lymphoprep™, routinely 5 to $10x10^8$ cells were obtained. These cells were resuspended in culture medium (M199 containing 10 mg/ml glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin) and 2.5 x 10^6 cells/well were seeded in 24 wells culture plates. After one hour the non-adherent cells were removed with medium, and cells were cultured in culture medium containing 0.1 µg/ml dexamethasone and 10% (v/v) human AB$^+$ serum. After 2 days the monocytes were

differentiated to macrophages and used for degradation studies.

Degradation of ^{125}I-t-PA by human macrophages.
Recombinant t-PA was labelled with ^{125}I using the iodogen method.[18] The macrophages were washed (3x with PBS containing 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mg/ml BSA, pH 7.4, 4°C), and incubated with 17 nM ^{125}I-t-PA with or without 100 nM GST-RAP and inhibitor in 300 µl M199, 10 mg/ml BSA, 0.01% (v/v) Tween 80, at 37°C in 5% CO_2/ 95% air. After one hour of incubation, cell media were collected and trichloroacetic acid (TCA) was added (final conc. 10% w/v). Non-degraded ^{125}I-t-PA was precipitated by centrifugation (10 min 15000 g). To eliminate the possibility that the cells might deiodinate rather than degrade ^{125}I-t-PA, free ^{125}I was extracted. To the 500 µl TCA soluble supernatant obtained, 5 µl 400 mg/ml KI and 25 µl H_2O_2 30% (v/v) were added. After 5 minutes the free iodine was extracted with 800 µl chloroform. The radioactivity of the remaining ^{125}I-tyrosine (and possibly ^{125}I-peptides) in the upper layer (5 min 15000 g) representing degraded ^{125}I-t-PA was determined. Data were corrected for radioactivity of the medium incubated in wells without cells.

The effect of dextran infusion in vivo in the rat.
Male Wistar rats (300 g) were anaesthetised with Nembutal (1 ml/kg i.p.) and Hypnorm/aqua (25 µl/rat i.m.). The arteria carotis and the vena femoralis were canulated with heparinized canules. A blood sample was taken from the a. carotis, and 2 ml of 0.9% NaCl or 6% dextran 70 in 0.9% NaCl (Macrodex) was infused via the vena femoralis in 45 minutes. Samples of blood were taken after 25 min and 45 min. Thereafter 500 µg/kg bw. recombinant t-PA was injected via the vena femoralis. Blood samples were taken on time= 1, 2, 3, 4, 5, 7, 10 and 15 minutes after injection. Haematocrit was measured at 0, 25, 45 and 60 min. All blood samples were diluted with 3.8% (w/v) trisodium citrate.$2H_2O$ (1 vol. to 9 vol. blood), kept on ice during the experiment, centrifugated (10 min 2000g), and plasma was frozen to -20 °C until analysis. Rat endogenous t-PA concentrations were measured using an Elisa specific for rat t-PA, [23] and recombinant t-PA was measured using an Elisa specific for human t-PA.[24]

Analysis of inhibition curves and t-PA clearance
Inhibition of the binding or degradation of labelled-t-PA will lead to a decreased value for labelled-t-PA binding or degradation expressed as a percentage of control. Isolated mannose receptor or cells were incubated with a concentration range of inhibitor and a nonsaturating concentration of labelled t-PA, the binding or degradation was measured and corrected for non-specific binding or degradation. The resulting inhibition curves were fitted as a sigmoid curve using nonlinear regression analysis with the computer program GraphPAD (ISI Software, Philadelphia, PA, USA), yielding half maximal inhibitory concentration (IC_{50}), and Hill-slopes. "Goodness of fit" was assessed by evaluating the actual distance of the measurements from the fitted line (no weighing).

Endogenous t-PA concentrations were corrected for the citrate dilution and the haematocrit measured at the corresponding sample time. The plasma concentration of the injected recombinant t-PA at zero time was calculated using the previously determined blood volume (7.3 ml blood/ 100 g rat [25]), the measured haematocrit and the citrate dilution. Using the above-mentioned computer program the t-PA clearance curves were fitted using a two-compartment model. Clearance was calculated by dividing the calculated initial concentration in plasma by the area under the clearance curve calculated from 0 to 15 minutes. Data are expressed as mean ± standard error of the mean (SEM), and statistical significance of differences was determined using the program SOLO 4.0 (BMDP Statistical software, Los Angeles, CA).

RESULTS

We tested the effect of dextran and various monosaccharides on the binding of t-PA to the isolated and immobilised human mannose receptor. As shown in Fig 1 the polymers of glucose, dextran 40 and dextran 70, inhibited t-PA binding with IC_{50} values of respectively 14 and 4 mg/ml. Though the name of the mannose receptor suggests that it only has an affinity for mannose-containing ligands, the receptor has a lower affinity for other carbohydrates. As shown in Fig 2 D-mannose, D-glucose and N-acetyl-D-galactosamine inhibited t-PA binding with decreasing affinities (IC_{50} respectively 2, 7 and 57 mg/ml). On a weight basis dextran 40 and 70 inhibited t-PA binding with an affinity comparable to that of D-glucose. We tested whether dextran was also able to inhibit mannose receptor-mediated degradation by human monocyte-derived macrophages. These cells express the mannose receptor and LRP. [18] In order to study mannose receptor-mediated degradation we upregulated the mannose receptor using dexamethasone and we used GST-RAP to inhibit LRP-mediated t-PA degradation. [18] As shown in Fig 3, the mannose receptor-mediated t-PA degradation is inhibited by dextran 40 and dextran 70 (IC_{50} respectively 7 and 2 mg/ml).

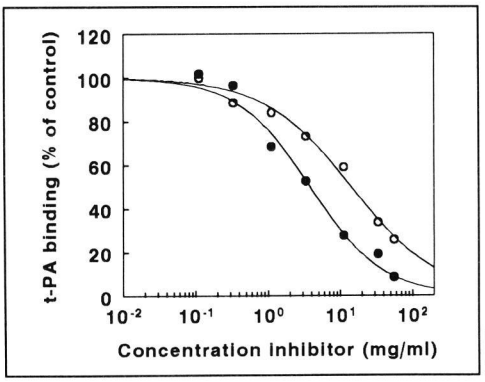

Fig 1 Inhibition of t-PA binding to the isolated human mannose receptor by dextran.
Isolated immobilised human mannose receptor was incubated with biotinylated t-PA and increasing concentrations of dextran. Residual binding was determined and expressed as a percentage of control. Data represent the mean of duplicates, curves were fitted from 100 to 0%. Dextran 70 (●) or dextran 40 (○).

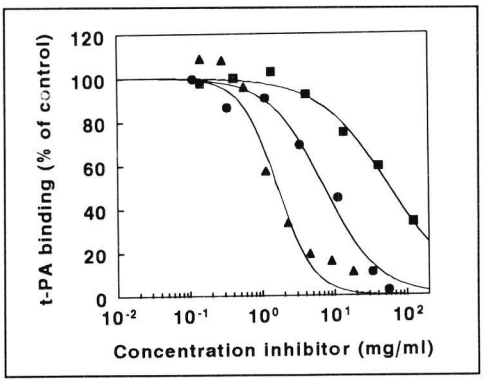

Fig 2 Inhibition of t-PA binding to the isolated human mannose receptor by monosaccharides.
Isolated immobilised human mannose receptor was incubated with biotinylated t-PA and increasing concentrations of monosaccharides. Residual binding was determined and expressed as a percentage of control. Data represent the mean of duplicates and curves were fitted from 100 to 0%. D-mannose (▲), D-glucose (●), N-acetyl-D-galactosamine (■).

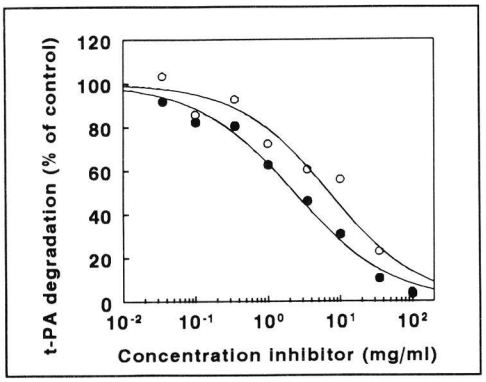

Fig 3 Inhibition of mannose receptor-mediated t-PA degradation by dextran.
Human monocyte-derived macrophages were incubated with radiolabelled t-PA and a range of dextran concentrations in the presence of 100 nM GST-RAP (mannose receptor-mediated degradation). Degradation was determined and expressed as a percentage of control. Data represent the mean of duplicates, curves were fitted from 100 to 0%. Mannan (1 mg/ml) inhibited t-PA degradation for 93.4%. Dextran 70 (●), dextran 40 (○).

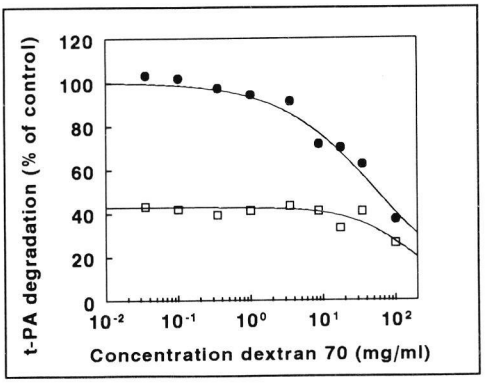

Fig 4 Inhibition of total and LRP-mediated t-PA degradation by dextran 70.
Human monocyte-derived macrophages were incubated with radiolabelled t-PA and a range of dextran 70 concentrations in the absence (total degradation (●)) or presence of 1 mg/ml mannan (LRP mediated degradation (□)). Degradation was determined and expressed as a percentage of control. Data represent the mean of duplicates; curves were fitted from 100% or from the level obtained in the presence of mannan without dextran to 0%.

To study whether LRP-mediated degradation by the macrophages was inhibited by dextran we looked at the effect of dextran 70 on total t-PA degradation (mediated by both LRP and the mannose receptor) and on degradation in the presence of an excess of mannan (mediated by LRP). As shown in Fig 4 total degradation was only partially inhibited by dextran 70 at concentrations that inhibited mannose receptor-mediated degradation completely (Fig 3). Dextran 70 did not affect LRP degradation at low and intermediate concentrations, only the highest concentration used (100 mg/ml) started to inhibit LRP-mediated degradation. Therapeutic amounts of dextran 70 reach plasma concentrations of about 10 mg/ml directly after infusion, after 5 hours the concentration is reduced to 6 mg/ml. [10] Figures 3 and 4 shows that at 10 mg/ml of dextran 70 approximately 60% of mannose receptor-mediated t-PA degradation was inhibited, while LRP mediated t-PA degradation was not affected.

We tested the effect of dextran 70 infusion on the plasma concentration of endogenous t-PA, and on the clearance of a bolus injection of t-PA *in vivo* in the rat. A schematic overview of the experiment is shown in Fig 5A. During and after infusion of NaCl the haematocrit remained at 44%, while in the dextran-treated rats the haematocrit increased during infusion to 54% (t=25 min) and then steadily decreased to 49% (t=60 min). As shown in Fig 5B and Table 1, the endogenous t-PA concentration decreased during and after NaCl infusion, while it increased during and after dextran infusion in most rats. On average the clearance of exogenous t-PA was inhibited for 33 ± 9% by dextran (0.56 ± 0.07 ml/min) compared to NaCl treatment (0.85 ± 0.07 ml/min) (Fig 5C). Both half-lives of t-PA were decreased (see Table 1), while the amount of t-PA cleared in each phase remained the same for both treatments (approximately 70 % was cleared in the α phase, and 30% in the β phase). The rats treated with dextran that had the highest endogenous t-PA concentrations, were also the rats that had the lowest clearance of exogenous t-PA (Fig 5D). We conclude that our data support the hypothesis that dextran inhibits t-PA clearance *in vivo*.

Table 1 The effect of dextran on plasma endogenous t-PA concentrations and exogenous t-PA clearance *in vivo* in the rat.

Summary and significance of the differences between the effect of 0.9% NaCl or 6% dextran 70 in 0.9% NaCl infusions (see Fig 5) on the endogenous t-PA concentration and exogenous t-PA clearance in the rat. Data are represented as mean ± SEM (n=5), the probability of difference between NaCl versus dextran treatment is calculated using the Mann-Whitney test.

Parameter	NaCl infusion	Dextran infusion	Probability value
endogenous t-PA t=25 min (%t=0)	53 ± 6	162 ± 33	0.009
endogenous t-PA t=45 min (%t=0)	54 ± 7	122 ± 35	0.009
t-PA clearance (ml/min)	0.85 ± 0.07	0.56 ± 0.07	0.047
t-PA clearance half-life α (min)	0.24 ± 0.08	0.53 ± 0.07	0.028
t-PA clearance half-life β (min)	2.3 ± 0.4	4.1 ± 0.7	0.117

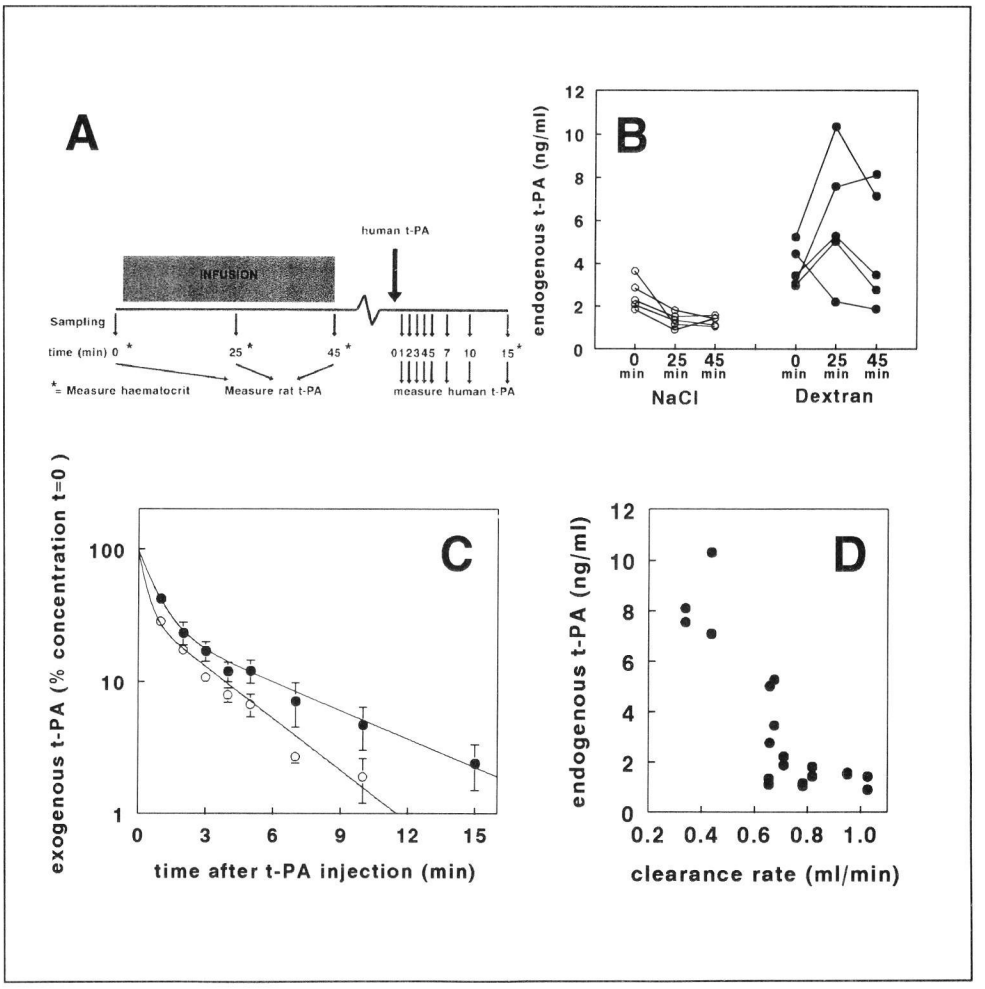

Fig 5 The effect of dextran on plasma endogenous t-PA concentration and exogenous t-PA clearance *in vivo* in the rat.
A schematic overview of the *in vivo* experiment is shown in Fig 5A. Before (t=0 min), during (t=25 min), and after (t=45 min) the infusion of 2 ml 0.9% NaCl (n=5) or 2 ml 6% dextran 70 in 0.9% NaCl (n=5) in the rat, plasma samples were taken and the endogenous t-PA plasma concentration was measured. Data were corrected for the haematocrit and citrate dilution, and are represented in Fig 5B (mean ± SEM; NaCl-treated rats open circles; dextran treated rats closed circles). After the infusion a bolus injection of 500 µg/kg bw. recombinant t-PA was given. At the time points indicated plasma samples were taken and human t-PA plasma concentration was measured. Data were expressed as a percentage of the calculated initial plasma concentration corrected for haematocrit and citrate dilution (11.0 µg/ml NaCl-treated rats, 12.2 µg/ml dextran-treated rats) and are shown in Fig 5C (lines: two-compartment model using the average clearance paramenters mentioned in Table 1, NaCl-treated rats (○); dextran-treated rats (●), mean plasma concentrations ± SEM). The correlation between clearance rates and endogenous t-PA concentration at t=25 min and t= 45 min of the rats is shown in Fig 5D.

DISCUSSION

Dextran has antithrombotic properties when used *in vivo* in man. [13-16] The antithrombotic effect of dextran is attributed to decreased coagulation by decreased platelet aggregation and adhesion, and to increased fibrinolysis by influencing fibrin structure. [10,13,26,27] However when at therapeutic concentrations dextran is added *in vitro* to normal blood or plasma, there is no reduction in platelet adhesiveness and no increased lysability of *in vitro* formed thrombi, while *in vitro* formed thrombi from blood obtained from patients receiving dextran were more easely lysed. [10] Thus dextran probably affects plasma concentrations of components of the coagulation and/or the fibrinolytic cascade *in vivo*.

The plasma levels of coagulation factors are not affected more than what would be expected from haemodilution except for the decrease in factor VIII:C and von Willebrand factor plasma concentrations which could contribute to the effects of dextran on platelet function. [10,28-30] Recently it has been shown that t-PA antigen and t-PA activity were increased during dextran infusions in surgical patients (by 18 and 43% respectively). PAI-1 activity was decreased by 19% probably by the increase in t-PA. [17] These findings are, however, not consistently found. Early studies showed that t-PA antigen as well as PAI-1 activity were significantly increased compared to preoperative values when measured one day after total hip replacement and dextran infusion. [23] No significant increase of t-PA activity was observed after dextran infusion, [28] and the desmopressin induced increase of t-PA antigen appeared not to be affected by dextran when studied in volunteers. [29] Whether these differences in observations were due to a difference in study design (such as blood sampling, patients versus volunteers, or number of participants) remains to be determined.

As dextran has been shown to be a possible ligand of the mannose receptor, [9] we hypothesized that inhibition of the mannose receptor-mediated t-PA clearance could contribute to the antithrombotic effect of dextran. In this study we tested whether dextran was able to inhibit mannose receptor-mediated t-PA degradation *in vitro* and *in vivo*. We showed that dextran inhibited binding of t-PA to the isolated immobilised mannose receptor. We also showed that mannose receptor-mediated t-PA degradation was inhibited (approximately by 60%) by dextran at therapeutic concentrations (10 mg/ml), whereas LRP mediated t-PA degradation was not. It appeared that dextran 70 had a 3 fold higher affinity than dextran 40 (6 fold higher when expressed in molar concentrations). Dextran 40 is clinically used in higher concentrations than dextran 70 (10% and 6% w/v solutions, respectively). The clinical plasma concentrations of dextran 40 are therefore higher, and based on our competition experiments the effects of both dextran forms on the t-PA clearance will be similar *in vivo*.

In vivo dextran treatment increased the plasma concentration of endogenous t-PA in the rat. This can be caused by stimulation of t-PA release and by inhibition of t-PA clearance. Therefore we also measured in the same rats the clearance of a bolus injection of recombinant t-PA. We found that the dextran treatment inhibited the clearance of exogenous t-PA by about 33%. The half-lives of both the rapid α-phase and the slower ß-phase were reduced by dextran, as observed previously with other inhibitors of mannose receptor-mediated t-PA clearance [6,32]

Dextran reduces thrombus formation and thrombus weight in a venous occlusion rabbit model. [33,34] Increased t-PA concentrations present during thrombus formation cause increased lysability of thrombi. [27,35,36] Recently it has been shown that even a doubling of plasma t-PA concentrations (within the physiological t-PA concentration range) is able to significantly reduce thrombus formation in a rat arterial thrombosis model. [37] Thus the increased t-PA concentrations during dextran infusion could contribute to the antithrombotic effect of dextran.

If partial inhibition of mannose receptor-mediated t-PA clearance already results in increased endogenous plasma t-PA concentrations, thereby reducing the risk of thrombosis in man, complete inhibition would even be more effective. A recently synthesized cluster mannoside with a high affinity for the mannose receptor [8] was able to fully inhibit mannose receptor-mediated t-PA clearance at plasma concentrations in the µg/ml range without showing any toxic effect in rats. [7] Would this compound be more efficient than dextran and be a better agent for preventing deep venous thrombosis in man? Further studies are required to provide evidence for this intriguing possibility.

In conclusion we showed that at therapeutic concentrations dextran is able to inhibit mannose receptor t-PA binding and to interfere with mannose receptor-mediated t-PA degradation by cells *in vitro*. We also showed that in rats dextran increases endogenous t-PA concentrations and inhibits t-PA clearance *in vivo*. As increased t-PA concentrations present during clot formation increase the thrombus lysability, the increased t-PA concentrations in plasma can contribute to the antithrombotic effect of dextran.

ACKNOWLEDGEMENT

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CHAPTER 9

General discussion

Regulation of tissue-type plasminogen activator concentrations by clearance via the mannose receptor and other receptors.

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SUMMARY

This review describes the identity, tissue distribution, ligands and functions of the mannose receptor and other tissue-type plasminogen activator (t-PA) clearance receptors like the low density lipoprotein receptor-related protein (LRP). The receptor binding sites of t-PA and the corresponding t-PA binding sites of the receptors are summarized, and recently developed inhibitors of t-PA clearance are described. The contribution of the receptors to the systemic clearance of t-PA and (patho)physiological conditions that may influence receptor expression, t-PA clearance and plasma t-PA concentrations are evaluated. Furthermore the potential use of mannose receptor inhibitors in thrombolytic and antithrombotic therapy is discussed.

Tissue-type plasminogen activator

Tissue-type plasminogen activator (t-PA) is one of the activators that is able to convert plasminogen into plasmin which cleaves fibrin into soluble degradation products. t-PA was purified from uterine tissue and found to be closely similar to the vascular plasminogen activator present in blood and in the human vascular wall. [1] It was isolated from the culture fluid of a melanoma cell line in 1981 and cloned and expressed in 1983. [2] High concentrations of active t-PA can rapidly activate the dissolution of blood clots. Infusions of recombinant t-PA are therefore successfully used in thrombolytic therapy. [3-5] New fibrinolytic agents such as t-PA mutants are currently in development. [4,6]

t-PA is a 69 kDa serine protease consisting of five domains: finger domain, growth factor domain, kringle 1, kringle 2, and a protease domain. Three potential N-glycosylation sites are present on the molecule, of which each is able to bear an oligosaccharide of a size comparable to that of a kringle domain (recently reviewed [7]). The oligosaccharide found on Asn 117 in kringle 1 is a high mannose-type oligosaccharide; Asn 148 in kringle 2 of t-PA may (type I t-PA) or may not (type II t-PA) be glycosylated with a complex-type oligosaccharide; and the protease domain bears a complex-type oligosaccharide on Asn 448. In addition a fucose unit is O-linked to Thr 61 in the growth factor domain of t-PA. Circulating plasma t-PA is synthesized and released by the endothelial cells that line the blood vessels. [8] The physiological plasma concentration of t-PA is low. Synthesis and blood clearance of t-PA as well as inhibition of t-PA by its inhibitor plasminogen activator inhibitor-1 (PAI-1) control the activity of t-PA in the blood. [9] High concentrations of PAI-1 in the blood lead to low t-PA activity. Low t-PA activity is associated with the risk of cardiovascular diseases such as myocardial infarction. [9,10]

Clot dissolution is a highly regulated process. [11] One intriguing phenomenon is that some coagulation factors (for example thrombin and factor Xa in combination with phospholipids) stimulate the endothelial cells to release large amounts of t-PA. [12,13] The concentration of active t-PA present during clot formation determines the lysability of the clot afterwards. [14,15] Recently it has been shown that very low dose t-PA infusions during thrombus formation (resulting in doubling of the physiological concentration of t-PA) reduces thrombus formation *in vivo* in the rat. [16] Instead of low dose infusions of t-PA, other ways to achieve higher active t-PA concentrations for prevention and therapy would be the administration of PAI-1 antagonists, [17] stimulators of t-PA synthesis and release [18] or t-PA clearance inhibitors.

This report describes the properties and the functioning of the mannose receptor and other clearance receptors. Furthermore, it evaluates their role in the regulation of the t-PA plasma concentration and discusses the potential therapeutic applications of t-PA clearance inhibitors.

Clearance of t-PA

The clearance of t-PA from the circulation is very rapid. In man both endogenous and recombinant t-PA have half-lives of approximately 5 minutes. [19-23] The liver is the main organ responsible for the clearance of t-PA. [19,21] In 1985 it was suggested for the first time that the mannose receptor is involved in the clearance of t-PA. [24] The first reports providing evidence for this process were published in 1988. [25-28] The 175 kDa mannose receptor isolated from bovine lung, [29] from human liver [30] and from human placenta, [31] has been shown to bind t-PA. Furthermore polyclonal antibodies against the human mannose receptor are able to inhibit endocytosis of t-PA by rat liver endothelial cells *in vitro*. [32] Recently we developed a panel of monoclonal antibodies against the human mannose receptor isolated from placenta. All monoclonal antibodies bind to the 175 kDa human mannose receptor in a Western blot and in an ELISA. Three of these monoclonal antibodies, which recognise the same epitope, are able to inhibit the binding of t-PA to the isolated mannose receptor and the mannose receptor-mediated t-PA degradation by human macrophages in culture. [33]

Inhibitors of the mannose receptor, however, only partially inhibit t-PA clearance. [26-28] After the identification of the mannose receptor as a t-PA clearance receptor, it took another four years before the α_2-macroglobulin receptor also called the low density lipoprotein receptor-related protein (LRP) was identified as another t-PA clearance receptor. [34-37] An inhibitor of the LRP also partially inhibited t-PA clearance. [38] Both the mannose receptor and LRP are about equally involved in the clearance of t-PA. [39,40] Administration of inhibitors of the mannose receptor and the LRP inhibited t-PA clearance each by approximately 60%, whereas a combination of these inhibitors inhibited clearance by approximately 90% in the rat. [40]

However, it cannot be excluded that besides the mannose receptor and LRP, other receptors were inhibited in these *in vivo* experiments. The 39 kDa receptor-associated protein (RAP) that was used to inhibit LRP-mediated t-PA clearance, also inhibits ligand binding to other receptors of the LDL receptor gene family such as the very low density lipoprotein (VLDL) receptor, epithelial glycoprotein 330 (gp330), and the low density lipoprotein (LDL) receptor. [41] Apart from LRP, both the VLDL receptor [42] and gp330 [37] also have been shown to bind t-PA at least when complexed to PAI-1 (t-PA-PAI-1). Inhibitors of the mannose receptor-mediated clearance may also inhibit the binding of ligands to other mannose-binding proteins (MBP) like serum amyloid P [43] and MBP-C [44] which have a ligand spectrum similar to that of the mannose receptor. It has been shown that serum contains a MBP that inhibits mannose receptor-mediated uptake by liver endothelial cells. [45] It is however not known whether t-PA binds to MBP.

Although t-PA clearance is already approximately 90% inhibited by a combination of mannose receptor and LRP inhibitors, other t-PA receptors may have a minor role in t-PA clearance. A fucose receptor has been suggested as mediating t-PA binding to hepatoma cells. [46] However, this could not be confirmed in another study [47] and no additional inhibition of t-PA clearance with fucosyl-BSA was found *in vivo*. [39] Some cell-associated molecules bind to the lysine binding site of t-PA (amphoterin, [48] gangliosides, α-enolase, [49] and other proteins [50]). These molecules are thought to play a role in the colocalization of t-PA and plasminogen and the formation of plasmin on the cell surface. Cell-associated proteins which do not bind t-PA in a lysine binding site or active site dependent way have also been found. [51-53] Furthermore it has been shown that t-PA is able to bind in a finger and kringle 2 domain-dependent manner to heparin. [54,55] Proteoglycans from vascular endothelial cells have been shown to bind t-PA. [57] Cell-bound proteoglycans have been shown to increase the binding of some ligands to the cell and

thereby the uptake by the LRP [56] and the VLDL receptor.[58] The contribution of the above-mentioned putative t-PA binding molecules to the clearance of t-PA is, however, still unknown.

The functioning of both the human mannose receptor and the LRP can be studied at the same time in cultured human monocyte derived macrophages. Inhibitors of the mannose receptor and the LRP inhibit t-PA degradation by macrophages each by approximately 50% and in combination by 95%. Furthermore, t-PA binding to these cells is partially inhibited by a lysine analog but this analog does not affect t-PA degradation. [59] We have shown that the effects of inhibitors studied in this human *in vitro* model are representative of the effects of inhibitors on the t-PA clearance *in vivo* in the rat. [60,61]

Tissue distribution of the mannose receptor and LRP

One of the above-mentioned monoclonal antibodies against the human mannose receptor was used in flow cytometry and immunohistochemistry to determine the cellular expression and the tissue distribution of the mannose receptor. [33,60,62,63] We have shown that isolated human monocytes and lymphocytes do not express the mannose receptor. Cultured human monocytes start to express the mannose receptor after a few days in culture. Expression of the mannose receptor as detected by this antibody is upregulated by dexamethasone and downregulated by lipopolysaccharide. [62]

By use of immunohistochemistry we have shown that the mannose receptor is expressed by only a few human cell types. In all vital organs resident tissue macrophages expressed the mannose receptor (including the alveolar macrophages, [63] Kupffer cells,[60] Hofbauer cells,[33] perivascular glial cells, and synovial lining cells [63]). Not all macrophages express the mannose receptor; the macrophages in the thymus medulla, [62] in the B cell areas of lymph nodes [62] and spleen, [63] and the microglial cells in the brain [63] were all mannose receptor-negative. In these studies the only non-mononuclear phagocytes expressing the mannose receptor in human tissue are liver sinusoidal endothelial cells, [60] spleen sinusoidal endothelial cells [63] and sperm cells. [63] In these studies it was also shown that the monoclonal antibody is specific for the 175 Kd mannose receptor; the antibody did not stain cells that express MBP like liver parenchymal cells [64] and lymphocytes[65] nor did it stain cells that express receptors with a configuration similar to that of the mannose receptor (DEC-205 [66] and phospholipase A2 receptor [67]).

In contrast to the mannose receptor, LRP is expressed in many more cell types such as neurons, astrocytes, epithelial cells, smooth muscle cells, fibroblasts, lipocytes, fibroblasts, chondrocytes, hepatocytes, syncytiotrophoblasts, Leydig cells, monocytes, macrophages, Kupffer cells, and Hofbauer cells. [68] gp330 is not expressed in the liver but only in kidney, lung and intestine.[69] The VLDL receptor is found in skeletal muscle, heart muscle and vascular endothelial cells. [70]

The ligands and functions of the mannose receptor

Alveolar macrophages are able to bind the lysosomal enzyme ß-glucuronidase in a mannan-inhibitable way. [71,72] Non-parenchymal liver cells are found also to endocytose similarly glycosylated lysosomal enzymes in a mannan-inhibitable way. [73,74] After the binding of the ligand to this mannan-inhibitable receptor, the ligand-receptor complex is internalised, the ligand dissociates from the receptor in the acidic environment of the endosome (pH 5-6) and the receptor is recycled to the membrane while the ligand is delivered to the lysosome. [75-77] The 175 kDa mannose receptor has been purified from extracts of macrophages, [78] placenta [79] and liver. [30] In 1990 the primary structure of the receptor was elucidated [80], and the characteristics of the individual mannose receptor domains were studied by using mutant receptors. [81-84]

An overview of the mannose receptor ligands is shown in Table 1 (see page 108). All mannose receptor ligands are also ligands for other receptors. Various (lysosomal) enzymes are cleared by the mannose receptor as well as the mannose-6-phosphate receptor or the galactose receptor. [85] The contribution of each receptor to the clearance of endogenous lysosomal enzymes can be different from the clearance of administrated isolated enzymes. [85] Some enzymes, for example renin, [93] consists of different isoenzymes and only a subpopulation (subtype B of renin) of the isoenzymes contains a mannose receptor recognition site. Therefore it is difficult to predict what inhibition of the mannose receptor would do to the concentrations of endogenous ligands *in vivo*. Until now only one study has shown that inhibition of the mannose receptor *in vivo* results in increased endogenous t-PA plasma concentrations.[61]

In the liver the mannose receptor functions mainly as a clearance receptor of ligands in the circulation. Some lysosomal enzymes are not degraded after uptake, instead they continue to function in the endothelial cell lysosome. [86,89] It has been shown that the mannose receptor-mediated endocytosis by liver endothelial cells is 3 to 7 times higher than by Kupffer cells. [115] This is also reflected in the *in vivo* uptake of t-PA where the endothelial cells contain six times more t-PA per mg cell protein than the Kupffer cells. [26] In contrast to healthy human cells, apoptotic human cells expose mannose and galactose residues on their surface. The mannose receptor is one of the receptors used in the liver by non-parenchymal cells to phagocytose apoptic cells. [105,106] In contrast to endocytosis, mannose receptor-mediated phagocytosis is more rapidly mediated by the Kupffer cells than by liver endothelial cells. [105]

In addition to the clearance of glycoproteins, the mannose receptor functions to mediate the phagocytosis of various pathogens. [98] Furthermore the mannose receptor is likely to be involved in antigen presentation, [116] foreign body-type giant cell formation, [117] the homing of lymphocytes to the spleen, [118] retinal phagocytosis [104] and sperm fertility. [119]

t-PA binding sites of the mannose receptor

Unlike the LRP ligands (see below) many mannose receptor ligands have been shown to inhibit each other's binding, though with different affinities. Studies with mutant t-PA and endo H treated t-PA have shown that the (tri-antennary) high mannose-type oligosaccharide present on kringle 1 of t-PA binds to the mannose receptor and mediates part of the t-PA clearance. [29,120] We have shown recently that the binding of t-PA to the mannose receptor is much stronger (500 fold) than the binding of two other ligands containing a similar high mannose-type oligosaccharide: ribonuclease B and ovalbumin. The complex-type oligosaccharides and the O-linked fucose in the growth factor domain of t-PA did not significantly contribute to this high affinity binding of t-PA. The affinity of the trypsin digest of t-PA was 500 fold lower than that of intact t-PA, whereas trypsin digests of ribonuclease B and ovalbumin had a 2-3 fold lower affinity than intact ribonuclease B and ovalbumin. This indicated that the high affinity of t-PA was not caused by a different type of oligosaccharide. We hypothesized that the conformation of the high mannose-type oligosaccharide is influenced by the protein part of t-PA in such a way that it has a higher affinity for the mannose receptor. Complex formation with PAI-1 or α_2-antiplasmin did not affect the affinity of t-PA for the mannose receptor. [31]

The mannose receptor (Fig 1) is a type I transmembrane protein consisting of a N-terminal cysteine rich domain, a fibronectin type II repeat, eight carbohydrate recognition domains (CRD), and a C-terminal transmembrane region and cytoplasmatic tail.[88] Using truncated mutant receptors it has been found that CRD 4, 5 and 7 are essential for the high affinity (Ki in the nM range) binding of mannan; mutant mannose receptors missing one or two of these domains have much lower affinities for mannan (Ki in the μM to mM range). CRD 4 is essential for efficient endocytosis, and CRD 6 and/or CRD 7 is essential for the release of the ligand at pH 5-6 .

The affinity of monosaccharides for the receptor is low (mM range) in the order D-mannose = L-fucose > N-acetylglucosamine ≥ D-glucose > D-galactose > N-acetylgalactosamine.[29,72,78] The affinity of polysaccharides for the receptor is much higher. Synthetic ligands like $mannose_{48}$-albumin (48 molecules mannose per molecule albumin) and $mannose_{104}$-polylysine have affinities in the nM range for the mannose receptor expressed on cells [77,122] while synthetic ligands containing less mannose residues like $mannose_5$-albumin [122] and polylysines containing two to four mannose residues[123] have affinities in the μM range. Isolated glycopeptides containing tri-antennary high mannose-type oligosaccharides have affinities in the μM range and glycopeptides containing di-antennary oligosaccharides are not detectably endocytosed by rat liver endothelial cells *in vitro*.[124] Tri-antennary oligosaccharides with long antennae are able to inhibit antigen presentation by macrophages in the nM range while tri-antennary oligosaccharides with shorter antennae have affinities in the μM range.[125]

The above-mentioned differences in affinity of the mannose receptor-ligand interactions suggest that the number of, and spacing between, the terminal saccharides determine the number of carbohydrate recognition domains the ligand can bind to and thereby its affinity for the mannose receptor. Recently, lysine-based cluster mannosides have been synthesized with two ($mannose_2$-$lysine_1$, M_2L_1) to six ($mannose_6$-$lysine_5$, M_6L_5) terminal α-D-mannose residues that are connected to a polylysine backbone with flexible elongated spacers. We have shown that all mannosides are able to inhibit t-PA and ribonuclease B binding to the isolated mannose receptor. The affinity of this series of mannosides continuously increases from 20 μM to 1 nM per mannoside with mannose valency increasing from two to six.[126]

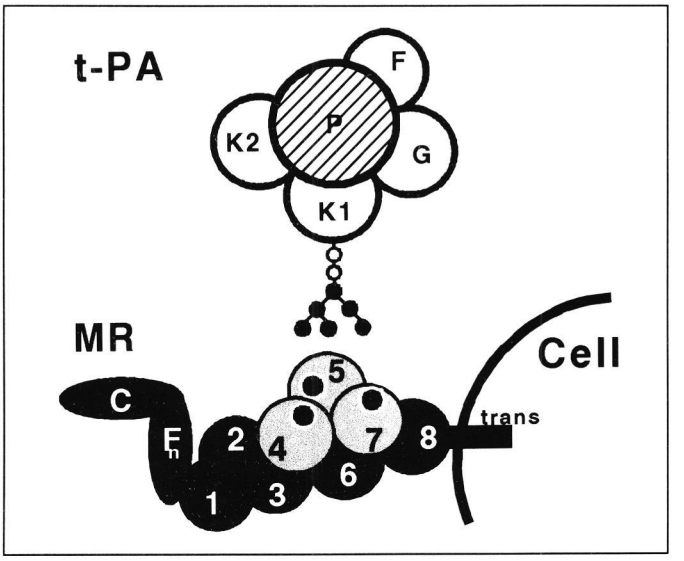
We have tested the same mannosides for their affinity towards isolated rat liver endothelial cells and cultured human macrophages that both express the mannose receptor. The affinity of the mannosides towards the mannose receptor expressed on cells show no species or cell-type differences. The mannosides M_5L_4 and M_6L_5 inhibit t-PA binding and degradation in the μM and the nM range, respectively. This difference in affinity is also found *in vivo*; 0.03 mg/rat or 0.3 mg/rat M_6L_5 inhibit t-PA clearance much stronger (respectively 50% and 60%) than 0.3 mg/rat M_5L_4 (40%).[60] A combination of 0.3 mg/rat M_6L_5 and 10 mg/rat RAP inhibit t-PA clearance by 90%.[40]

In conclusion: t-PA binds with its tri-antennary high mannose-type oligosaccharide with high affinity to the mannose receptor. This binding is completely blocked by mannose and by poly-mannosides. The affinity of the inhibitors increases with increasing mannose valency. The high affinity of t-PA for the mannose receptor suggests that the high mannose-type oligosaccharide of t-PA interacts with more than one carbohydrate recognition domain of the mannose receptor. A simple model for the interaction between the mannose receptor and t-PA is shown in Fig 1.

Fig 1

Schematic representation of the putative interaction of t-PA with the mannose receptor.

Mannose receptor (MR) with the N-terminal cysteine rich domain (C), fibronectin type II repeat (F_n), eight carbohydrate recognition domains (1-8) with the mannose binding sites (● in 4, 5 and 7), and a C-terminal transmembrane region and cytoplasmatic tail. t-PA with the finger domain (F), growth factor domain (G), kringle 1 (K1) containing a high mannose-type oligosaccharide with 2 N-acetyl-glucosamine residues (○) and at least 6 mannose residues (●), kringle 2 (K2), and the protease domain (P).



The ligands and functions of the LDL receptor family

The LRP is a member of the LDL receptor family, [127] and is involved in the clearance of a lot of different ligands: proteinases, inhibitors and their complexes with proteinases, and the lipoproteins LDL and apolipoprotein E (apo E) or lipoprotein lipase (LPL) enriched VLDL (apo E-VLDL, LDL-VLDL) (see Table 1, for reviews see [41,107,128-131]). No other functions than uptake and degradation of its ligands have been described for the various members of the LDL-receptor family. LRP is a very large type I transmembrane receptor consisting of two subunits of 515 kDa and 85 kDa, respectively. RAP is frequently used in inhibition studies to demonstrate involvement of the LRP, [132] but it has later been show that RAP also inhibits ligand binding to gp330 and the VLDL receptor. [41] gp330 is a receptor analogous to the LRP. It has a similar size and ligand spectrum as the LRP including the ligands t-PA-PAI-1, [37] urokinase-PAI-1 (uPA-PAI-1) [133] and thrombin-PAI-1. [110] As gp330 is not expressed in the liver or vascular endothelial cells [69] it is not likely to be involved in t-PA clearance. The VLDL receptor is a 130 kDa protein which does not recognise activated (fast form) α_2-macroglobulin (α_2 M*) but does mediate internalisation of pro-uPA, uPA-PAI-1, LPL, [134] and apo E-VLDL.[58] In contrast to the LRP [133] the VLDL receptor is expressed by vascular endothelial cells. [70] t-PA-PAI-1 binds to the VLDL-receptor, [42] and it has been shown that vascular endothelial cells degrade t-PA probably after complex formation with PAI-1, [135,136] and that degradation by these cells could be inhibited by RAP.[136] Thus the VLDL receptor on endothelial cells might contribute to the plasma clearance of t-PA.

t-PA binding sites of LRP

Some controversy exists whether the LRP is able to mediate binding of free t-PA. t-PA does not bind to the LRP in a ligand blot, while t-PA-PAI-1 does. [36,137] On the other hand t-PA can bind to the isolated immobilised LRP [109] and inactivated t-PA, unable to interact with PAI-1, is still degraded by the LRP expressed on hepatoma cells [36,138] and human macrophages.[59] It cannot be excluded that t-PA binds in an active site-independent manner to a third molecule that is lost during ligand blotting and that t-PA, after binding to this molecule, is taken up via the LRP expressed on cells.

Table 1. An overview of the ligands of the mannose receptor and of LRP.

Mannose receptor ligands	LRP ligands
enzymes	**enzymes**
tissue-type plasminogen activator (t-PA) (see text)	tissue-type plasminogen activator (t-PA) (see text)
ß-glucuronidase [71,72]	urokinase (u-PA) [41,107]
N-acetyl-ß-glucuronidase [73]	pro-urokinase (pro-u-PA) [108,109]
α-mannosidase [73]	lipoprotein lipase (LPL) [41,107]
ß-galactosidase [74,85]	kallikrein [108]
glycosylasparaginase [86]	**inhibitors**
hyaluronidase [87]	plasminogen activator inhibitor-1 (PAI-1) [110]
arylsulfatase A [85]	tissue factor pathway inhibitor (TFPI) [41]
salivary amylase [88]	**enzyme-inhibitor complexes**
myeloperoxidase [89]	t-PA-PAI-1 [41,107] u-PA-PAI-1 [41,107] and thrombin-PAI-1 [111]
dopamine-ß-hydroxylase [90]	α_2-macroglobulin-proteinase (fast form) α_2M, α_2M^* [41,107]
acetylcholine esterase [91]	pregnancy zone protein-proteinase [41,107]
tissue kallikrein [92]	elastase-α_1-antitrypsin [41]
renin (subtype B) [93,94]	thrombin-antithrombin III [112]
glycoproteins	thrombin-heparin cofactor II [112]
B-cell modified α_2-macroglobulin (slow form) [95]	u-PA-protease nexin I [41]
C-terminal propeptide type I procollagen [96]	**lipoproteins**
uteroferrin [97]	low density lipoprotein (LDL) [113]
organisms	apolipoprotein E enriched ß-very low density lipoprotein (apo E-ß-VLDL) [41,107]
Kliebsella Pneumoniae [98]	LPL enriched VLDL (LPL-VLDL) [41,107]
Escherichia Coli [98]	LPL enriched ß-VLDL (LPL-ß-VLDL) [41,107]
Mycobacterium Avium [98]	**matrix proteins**
Mycobacterium Tuberculosis [98,99]	thrombospondin 1 [41] and 2 [114]
Pseudomonas Aeroginosa [98]	**toxins and viruses**
Aspergillus Fumigatus [98]	*Pseudomonas* exotoxin A [41,107]
Candida Albicans [98]	minor-group human rhinovirus [41,107]
Candida Krusei [98]	**other ligands**
Cryptococcus Neoformans [98]	apolipoprotein E (apo E) [41,107]
Saccharomyces Cerevisae [100]	lactoferrin [41,107]
Pneumocytis Carinii [98]	receptor associated protein (RAP) [41,107]
Trypanosoma Cruzi amastigotes [101]	
Leishmania Donovani [98]	
other ligands	
ricin [102]	
bee venom phospholipase A [103]	
photoreceptor outer segments [104]	
apoptotic lymphocytes/apoptotic bodies [105,106]	

The LRP binds to its ligands with different sites on the receptor. Although RAP seems to inhibit binding of all ligands, not every ligand competes with all other ligands for binding to the LRP. LPL-VLDL, apo E-VLDL, lactoferrin nor α_2M* are able to inhibit t-PA-PAI-1 degradation by human fibroblasts. [37] t-PA and pro-u-PA have different binding sites since the isolated immobilised LRP binds t-PA and this binding is not inhibited by pro-u-PA. [109] Using a mutant LRP (amino acids 836-2501) it was found that the binding sites of t-PA, t-PA-PAI-1 and uPA-PAI-1 are located on region II of LRP. [137] This mutant receptor is not able to interact with α_2M*, while in another study an isolated LRP fragment (amino acids 776-1399) has been found to bind the light chain of α_2M (binding domain similar to α_2M*) and u-PA-PAI-1. [139] uPA-PAI-1 binding to the isolated LRP is inhibited by uPA-PAI-1, pro-u-PA, uPA, t-PA-PAI-1 and active or latent PAI-1 with high affinity, while t-PA inhibits uPA-PAI-1 binding with a very low affinity. [108,110] This indicates that u-PA-PAI-1 has overlapping binding sites with pro-u-PA, PAI-1 and t-PA-PAI-1 but not with t-PA.

The t-PA growth factor and finger domain appear to mediate binding of t-PA and t-PA-PAI-1 to the LRP. Inhibition studies with isolated t-PA domains show that a fragment containing the finger and growth factor domain of t-PA is able, and mutant t-PA lacking the finger and growth factor domain is unable, to inhibit t-PA and t-PA-PAI-1 degradation by Novikoff hepatoma cells. [47,141] Mutant t-PA lacking the finger and growth factor domain has also been shown to be unable to inhibit t-PA binding to smooth muscle cells. [135] The clearance of t-PA mutants is delayed when they have deletions in the finger or growth factor domain indicating the involvement of these domains in the binding of t-PA to clearance receptors *in vivo*. [142] A t-PA mutant consisting only of the kringle 2 and protease domain ("reteplase") has a low affinity for isolated parenchymal cells and RAP did not significantly affect the plasma clearance of reteplase. [140] Compared to t-PA, reteplase is also less efficiently degraded by vascular endothelial cells, the degradation was RAP inhibitable. [136] Though only a small amount of reteplase is cleared by the liver, it is still taken up by the liver in a RAP inhibitable way, which indicates that the liver-mediated clearance of reteplase and/or complexes of reteplase with PAI-1 is mediated by the LRP. [140]

It appears that the LRP binding sites of t-PA and t-PA-PAI-1 are only partially overlapping. RAP binds in a Ca^{2+}-dependent and a Ca^{2+}-independent way to the various ligand binding sites of LRP. [132] t-PA binding by hepatic cells is Ca^{2+}-dependent [143,144], while the binding of t-PA-PAI-1 is not. [145] It has been shown that Fab fragments against Ca^{2+}-dependent binding sites of LRP are able to inhibit u-PA-PAI-1 and pro-u-PA binding to the isolated LRP, while they are not able to inhibit t-PA-PAI-1 binding. [146] Binding studies with mutants of t-PA revealed that t-PA lacking the growth factor domain binds in a Ca^{2+} independent way, while t-PA lacking the finger domain binds in a Ca^{2+} dependent way to rat liver parenchymal cells. [144]

In the above-mentioned studies the affinities of t-PA and t-PA-PAI-1 for LRP expressed on intact cells were in the range of 1-30 nM. Compared to u-PA or PAI-1, u-PA-PAI-1 has a higher affinity for the LRP [110]; similarly, compared to t-PA, t-PA-PAI-1 has a higher affinity for human hepatocytes [147] and is degraded faster than t-PA by rat novikoff hepatoma cells. [141] The clearance of t-PA-PAI-1 is twice as fast as the clearance of free t-PA and six times faster than the clearance of free PAI-1 in an isolated perfused rat liver. [148] However it also has been found that t-PA is cleared faster in healthy human volunteers with low levels of PAI-1 activity than in volunteers with high PAI-1 activity. [149] Thus it appears that active t-PA is cleared faster than t-PA-PAI-1 in man. A similar conclusion was reached by Chandler et al. [150]

In conclusion: the LRP binding site of t-PA not complexed to PAI-1 is partially overlapping with the t-PA-PAI-1 binding site. t-PA and t-PA-PAI-1 probably bind with the finger and/or growth factor domain to the LRP. PAI-1 increases the affinity of t-PA for the LRP probably by binding of the PAI-1 moiety to a PAI-1 binding site on the LRP. The LRP binding sites of t-PA and pro-u-PA are different. The binding sites of t-PA-PAI-1 and uPA-PAI-1 are partially overlapping probably by the PAI-1 binding site, and the binding sites of pro-uPA and uPA-PAI-are partially overlapping probably by the u-PA binding site. PAI-1 also increases the affinity of u-PA for the LRP. All these binding sites are likely to be localized on the second cluster of the complement-type cysteine-rich repeat (amino acids 836-1399) of LRP.

Regulation of the mannose receptor and LRP

Upon maturation from monocytes (which do not express the mannose receptor) to macrophages the mannose receptor is expressed. [59,62,151] Interleukin-4, [152] interleukin-13, [153] prostaglandin E2 [154] and dexamethasone [59,62,155,156] upregulate the mannose receptor expression on macrophages. Bacillus Calmette Guerin, [158] lipopolysaccharide, [59,62,116,159]oxidant, [160]proteolysis, [161]γ-interferon, [162]and a combination of interleukin-1 and tumour necrosis factor-α [116] all downregulate mannose receptor-mediated ligand uptake by macrophages. Only for γ-interferon it has been shown that it downregulates mannose receptor biosynthesis. The downregulation by γ-interferon can be counteracted on the mRNA level by prostaglandin E2 [162] and dexamethasone. [163] These studies were all performed on macrophages *in vitro*. Recently, we have shown that up- or downregulation of the mannose receptor also occurs *in vivo* in man. [63] Upregulated mannose receptor expression is found in a foreign body reaction in the synovium, and downregulated mannose receptor expression is found on macrophages in human tissue affected by diseases such as cancer, tuberculosis and rheumatoid arthritis. [63]

Little is known about the regulation of mannose receptor expression by liver endothelial cells but it might be regulated differently from macrophages. It has been shown that tumour necrosis factor-α and interleukin-1ß do not downregulate but instead upregulate the uptake of mannan in liver endothelial cells *in vitro*. This was not caused by upregulation of receptor expression but by upregulation of the endocytotic efficiency. [164] The mannose receptor expression on liver endothelial cells increases shortly after birth and decreases during ageing in the rat. [165] Furthermore glucose has been shown to downregulate mannose receptor expression on macrophages, [166] and high plasma glucose levels decrease the clearance of mannose receptor ligands in diabetic rats. [90,167,168]

Upon differentiation from monocytes (which do express the LRP) to macrophages the LRP is upregulated. [59] Activation by Bacillus Carmette Guerin and lipopolysaccharide downregulates the LRP expression on macrophages [59,158] while dexamethasone has no effect. [59] Dexamethasone upregulates the LRP on hepatoma cells. [169] Furthermore it has been shown that insulin upregulates the LRP on adipocytes [170] and on hepatoma cells. [171]

Conditions that may influence systemic t-PA clearance in vivo.

The expression of the mannose receptor and LRP on cells in tissues [63,68] may play an important role in regulating the local concentrations of t-PA, but this remains to be shown. The liver plays a major role in the clearance of t-PA from the blood. [19,21] The clearance by the liver is very efficient and not easily saturated. Exercise reduces the liver blood flow and increases thereby endogenous t-PA concentrations, while increase of the liver blood flow has no strong effect on t-PA concentrations. [22,23,150] The importance of efficient clearance of t-PA by the liver in maintaining homeostasis becomes evident in chronic liver disease where severe bleeding complications often are found. In this situation the concentration of t-PA

increases strongly while the PAI-1 concentration increases less strongly, [173] or even decreases. [174]

Some conditions have been shown to affect t-PA concentrations and have the same influence on the concentrations of other ligands of the mannose receptor. Increased levels of t-PA antigen and mannose receptor ligands are found under the following circumstances: ***liver disease*** (t-PA, [173,174] N-acetyl-ß-glucosaminidase, ß-glucuronidase [175]), ***gender*** and ***age*** (male > female and increase with age, t-PA, [176] ß-glucuronidase, N-acetyl-ß-glucosaminidase [177,178]), ***pregnancy*** (t-PA, [179,180] ß-glucuronidase and N-acetyl-ß-glucosaminidase [181,182]), ***high body mass index*** (t-PA, [183] N-acetyl-ß-glucosaminidase [178]), and ***obesity*** (t-PA, [180] N-acetyl-ß-glucosaminidase and α-D-mannosidase [184]). One study demonstrated that hyperlipidaemic rats have a decreased clearance of t-PA. [185] Whether in all above-mentioned conditions t-PA clearance is really affected remains to be shown.

A more complicated condition where both mannose receptor and LRP mediated t-PA clearance could be influenced is ***diabetes***. Diabetic rats have a decreased mannose receptor mediated clearance and increased plasma concentrations of the mannose receptor ligand dopamine ß-hydroxylase. [90,167,168] Insulin has been shown to upregulate LRP expression on adipocytes and hepatoma cells. [170,171] In diabetes type I patients increased concentrations of N-acetyl-ß-glucosaminidase and ß-glucuronidase [186-188] have been found. Diabetes type 1 patients have decreased [189,190] or increased [191] t-PA antigen concentrations. t-PA antigen is increased in diabetes type I patients before insulin treatment and decreased to below control values after insulin treatment. [192] In diabetes type II patients t-PA antigen positively correlates with insulin resistance. [193,194] In these patients both t-PA antigen [194,195] and N-acetyl-ß-glucuronidase [186] are increased and decreased concentrations are observed with glycemic control. [186,195] However, it remains to be determined whether mannose receptor and/or LRP-mediated t-PA clearance is influenced in diabetic type I and type II patients before and after insulin treatment.

Possible use of clearance inhibitors in thrombolytic and antithrombotic therapy

In thrombolytic therapy high doses of recombinant t-PA are used since it is cleared so fast from the circulation. Mutants of t-PA that have a lower affinity for the clearance receptors would require a lower dose for equal efficiency. Different mutants have been synthesized and tested for their efficiency compared to recombinant t-PA. Few mutants having a lower clearance rate retained the fibrin specificity and plasminogen activating capacity. These are currently being tested for their efficacy in thrombolytic therapy. [4,6,142,196] Beside mutants of t-PA, inhibitors of clearance may be used in combination with t-PA to diminish the dosage of t-PA necessary for thrombolytic therapy.

An additional effect of reducing the clearance of the plasminogen activator used in thrombolytic therapy would be to diminish the side effect of bleeding complications. High concentrations of t-PA can induce systemic plasminogenolysis and increased bleeding complications. [197] t-PA is a high clearance drug which means that the clearance is largely dependent on the liver blood flow. [172] Myocardial infarct patients can have a decreased liver blood flow and thereby they may be more susceptible to overdosage and thus to bleeding complications. [22,172] Mutants of t-PA have a lower clearance because they bind with a lower affinity (or not at all) to the liver receptors. Clearance inhibitors reduce t-PA clearance by competing with t-PA for liver receptors. The clearance of low clearance drugs is less influenced by the liver blood flow. Thus use of low clearance t-PA mutants or coadministration of t-PA clearance inhibitors with a lower dose of t-PA may diminish the differences in clearance and thus t-PA plasma concentrations between patients with a high and low liver blood flow and may reduce the risk of t-PA overdosing in myocardial infarction patients.

An additional potential use of clearance inhibitors in therapy would be the increase of endogenous t-PA concentrations. Since a low increase in the t-PA concentration present during clot formation already results in decreased thrombus formation, [16] such inhibitors may be used without exogenous t-PA in the prevention of thrombosis. A drug that may exert its antithrombotic effect by inhibition of t-PA clearance is dextran. Dextran is used as a plasma volume expander, as a blood substitute and as a prophylactic agent against deep venous thrombosis in surgery. [198,199] Dextran increases t-PA antigen concentrations and even more strongly increases the t-PA activity in patients. [200] As dextran is a glucose-polymer it is a potential inhibitor of mannose receptor-mediated t-PA clearance. We showed [63] that dextran inhibits the binding of t-PA to the isolated mannose receptor. At therapeutic concentrations dextran partially (60%) inhibits mannose receptor-mediated but not the LRP-mediated t-PA degradation by human macrophages. Furthermore we showed in the same report that an infusion of dextran increases endogenous t-PA concentrations in plasma by partially (33%) inhibiting t-PA clearance in rats. Thus the inhibition of t-PA clearance by dextran resulted in increased endogenous t-PA concentrations which may explain the antithrombotic effect of dextran in man. Since patients can be treated for days with dextran and dextran does not appear to have toxic side effects (beside immediate allergic reactions, or bleeding complications at a very high dosage, [198,199]) the inhibition of mannose receptor-mediated t-PA clearance may be a safe prophylactic therapy against thrombosis.

A recently developed cluster mannoside is able to inhibit t-PA clearance by 60% apparently without toxic effects in rats. [40] This compound could be more efficient than dextran and may have a stronger effect on endogenous t-PA concentrations and thus could be a better agent than dextran for preventing deep venous thrombosis in man. Further studies are required to provide evidence for this intriguing possibility of a new type of antithrombotic therapy.

Conclusion

The plasma clearance of t-PA is mainly mediated by the mannose receptor and the LRP expressed in the liver. The high mannose-type oligosaccharide of t-PA binds to the mannose receptor; the growth factor and finger domain are involved in the binding of t-PA to the LRP. The expression of these receptors in the liver possibly influences the clearance of t-PA under (patho)physiological conditions and thereby the plasma concentration of endogenous t-PA. Inhibitors of the (mannose receptor-mediated) clearance are of potential interest in thrombolytic and antithrombotic therapy.

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SUMMARY

The mannose receptor, localization and role in the clearance of tissue-type plasminogen activator.

After damage to a blood vessel a blood clot is formed (coagulation) and after tissue repair the clot is degraded (fibrinolysis). Coagulation and fibrinolysis are controlled by production of enzymes and enzyme inhibitors. The balance between these processes determines whether the clot is formed or degraded. Tissue-type plasminogen activator (t-PA) is an enzyme which converts plasminogen into plasmin, which in turn converts the insoluble network of the blood clot, fibrin, into soluble fibrin degradation products. Plasma t-PA is continuously synthesized and secreted by the endothelial cells that line the blood vessels, and continuously degraded (clearance) by the liver.

The clearance of t-PA by the liver is very efficient; within 5 minutes half of an injected dose of t-PA has disappeared from the plasma. The clearance of t-PA by the liver is a receptor-mediated process. t-PA binds to receptors expressed on the surface of cells in the liver. After binding the receptor-t-PA complex is internalized, t-PA is degraded in the cell and the receptor is recycled to the cell surface where it again can bind t-PA. The mannose receptor and the α_2-macroglobulin receptor (also called the low density lipoprotein receptor-related protein (LRP)) are mainly responsible for the clearance of t-PA by the liver. When a clot is formed t-PA is incorporated into the clot. The more t-PA is incorporated the higher the lysability of the clot. A high concentration of t-PA shifts the balance of coagulation and fibrinolysis towards fibrinolysis. Therefore recombinant t-PA is successfully used as a thrombolytic drug for instance after myocardial infarction and low dosage infusion of t-PA may be useful in antithrombotic therapy.

In this thesis we hypothesized that inhibitors of the mannose receptor-t-PA binding might decrease the clearance of t-PA. By inhibition of the clearance it might be possible to increase the efficacy of exogenous and endogenous t-PA. These inhibitors could thus be useful drugs in thrombolytic and antithrombotic therapy. The aim of this study was to extend our knowledge of the mannose receptor in order to develop efficient mannose receptor inhibitors. These inhibitors may in the future be used to treat thrombotic (and other) diseases.

Study design

To evaluate the human mannose receptor-t-PA interaction and to characterize the efficacy of inhibitors we developed two *in vitro* assays (chapter 1 and 2). Monoclonal antibodies (mAb) against the human mannose receptor were developed to specifically study the mannose receptor that is able to bind t-PA (chapter 3). To assess the possible role(s) of the mannose receptor in man, these mAb were used to visualize the mannose receptor expression on cells *in vitro* and in human tissues under physiological and pathological conditions (chapter 4 and 5). The inhibitory efficacy of newly synthesized mannose receptor ligands and the antithrombotic agent dextran was tested *in vitro* and *in vivo* (chapter 6,7 and 8). In the last chapter (chapter 9) the results of this study are discussed and the regulation of plasma t-PA concentrations by receptor-mediated clearance is reviewed.

In vitro models of the interaction between the human mannose receptor and t-PA

The mannose receptor contains multiple carbohydrate recognition domains. The more carbohydrate binding sites the ligand binds to, the higher the affinity of the ligand. Previous research showed that the high mannose-type oligosaccharide (a carbohydrate polymer with three terminal mannose residues) of t-PA binds to the mannose receptor. In chapter 1 we further characterized the interaction of t-PA with the isolated human mannose receptor. The mannose receptor was isolated from human placenta, and the binding of t-PA and t-PA variants was measured in a microtitre plate binding assay. Compared to two other glycoproteins, ribonuclease B and ovalbumin, that also contain a high mannose-type oligosaccharide, t-PA binds with a much higher (500 times) affinity to the human mannose receptor. The binding of t-PA to the mannose receptor was specific since the binding was fully inhibited by mannan (a polymer of mannose) or by polyclonal antibodies against the mannose receptor. Since a variant of t-PA that did not contain oligosaccharides did not inhibit the binding of t-PA to the mannose receptor, it is unlikely that protein-protein interactions contribute significantly to the high affinity binding of t-PA. Complex formation of t-PA with plasminogen activator inhibitor type 1 (PAI-1) or α_2-antiplasmin did not affect the affinity of t-PA for the mannose receptor. Furthermore the complex-type oligosaccharides or the O-linked fucose residue of t-PA were not involved in the high affinity of t-PA.

The protein moieties of t-PA, ribonuclease B and ovalbumin were fully digested with trypsin, an enzyme that does not digest the oligosaccharides of the glycoproteins. The trypsin digest of t-PA had a 500-fold lower affinity than intact t-PA, whereas trypsin digests of ribonuclease B and ovalbumin only had a 2 to 3 times lower affinity than the intact glycoproteins. This indicated that the high mannose-type oligosaccharide of t-PA did not bind differently from that of ribonuclease B or ovalbumin to the mannose receptor. It is likely that the conformation of the high mannose-type oligosaccharide of t-PA is influenced by the protein core in such a way that the oligosaccharide binds to more carbohydrate recognition domains of the mannose receptor and thus causes the high affinity of intact t-PA for the mannose receptor. The binding assay with isolated human mannose was in further studies used as an *in vitro* model to study the efficacy of inhibitors of the mannose receptor-t-PA binding (see chapter 3,6,7,8)

In chapter 2 we characterized the receptor systems involved in the binding, association and degradation of t-PA by human macrophages in culture. Monocytes were isolated from human buffy coats and cultured. Within two days the monocytes were differentiated to macrophages. At 4°C t-PA bound to macrophages with high (Kd = 1-5 nM) and low (Kd > 350 nM) affinity. The low affinity binding sites were inhibited with the lysine analog 6-aminohexanoic acid (6-AHA). At 37°C the macrophages internalised and degraded t-PA. This process was partially inhibited by mannan or a monoclonal antibody against the mannose receptor (see chapter 3). The non-mannan-inhibitable degradation of t-PA was inhibited by receptor associated protein (RAP), an inhibitor of LRP. Neither inactivation of t-PA nor the addition of 6-AHA influenced the degradation of t-PA. The degradation of t-PA by monocytes was not mannan-inhibitable. The mannose receptor- and LRP-mediated t-PA degradation was upregulated during the differentiation of monocytes to macrophages. Dexamethasone upregulated only the mannose receptor-mediated t-PA degradation. Lipopolysaccharide downregulated both mannose receptor-mediated and LRP-mediated t-PA degradation. We concluded that human macrophages use two independently regulated receptors, namely the mannose receptor and LRP, for the uptake and degradation of t-PA. Since these receptors are mainly responsible for the clearance of t-PA in the liver, cultured human macrophages are a suitable *in vitro* model for evaluating inhibitors of t-PA clearance. Therefore the macrophages were used in further studies to assess the efficacy of inhibitors to inhibit the mannose receptor-mediated t-PA degradation (see chapter 3,7,8)

Development and applications of monoclonal antibodies against the human mannose receptor

A set of five monoclonal antibodies (mAb) was generated against the mannose receptor isolated from human placental tissue and characterized (chapter 3). All mAb specifically recognised the 175 kDa mannose receptor in a crude placenta extract, as shown in Western blot analysis. By use of immunohistochemistry we showed that in human placenta only the Hofbauer cells (fetal macrophages) express the mannose receptor. Cross-competition experiments indicated that the mAb bound to at least two different epitopes on the isolated mannose receptor. One of these epitopes was located closely to the t-PA binding site, since the mAb directed against this epitope strongly inhibited the binding of t-PA to the isolated mannose receptor and the mannose receptor-mediated degradation of t-PA by cultured human macrophages.

These mAb were used to study the expression of the mannose receptor on isolated cells and human lymphoid tissues (chapter 4). Isolated human monocytes and lymphocytes did not and cultured macrophages did express the mannose receptor as determined with flow cytometry. The mannose receptor was upregulated on dexamethasone-treated macrophages, and downregulated on lipopolysaccharide-treated macrophages. The mannose receptor expression of a number of cell types involved in the immune system was evaluated by the use of immunohistochemistry (with mAb 15-2). Monocytes, lymphocytes, granulocytes and Langerhans cells were mannose receptor-negative, tissue macrophages in thymus, lymph nodes, bone marrow and skin were mannose receptor-positive. Interestingly the macrophages in the thymus medulla and T cell areas of the lymphodes were mannose receptor-positive, whereas the macrophages in thymus cortex and B cell areas of lymph nodes were mannose receptor-negative. Monocytes can differentiate into antigen-presenting cells (dendritic cells). It has been shown that the mannose receptor is involved in the uptake of antigens by dendritic cells *in vitro*. When these cells are stimulated to present the antigen to T cells, the mannose receptor is downregulated. In the thymus cortex and the B cell areas antigen is presented by dendritic cells, which could explain the absence of mannose receptor-positive cells in these areas.

Besides mediating the clearance of glycoproteins that contain high mannose-type oligosaccharides in the liver, the mannose receptor has been shown to be involved in several other processes. *In vitro* experiments have shown that the mannose receptor is involved in the macrophage complement-independent phagocytosis of pathogens such as Candida and Tuberculosis, and probably is involved in the fusion of macrophages into foreign body-type giant cells, the homing of lymphocytes to the spleen, and sperm fertility. The mannose receptor expression correlates to the type of macrophage activation *in vitro*; the T-helper type 1 cytokine γ-interferon activate the macrophage in such a way that the mannose receptor expression is downregulated, whereas the T-helper type 2 cytokines interleukin-4 and interleukin-13 activate the macrophage in such a way that the mannose receptor is upregulated (also called immunosuppression or alternative activation).

In chapter 5 we used one of the mAb (mAb 15-2) to evaluate the tissue distribution of the human mannose receptor under physiological and pathogenic conditions. Only a few cell types expressed the receptor under physiological conditions: resident macrophages in all tissues, liver and spleen sinus endothelial cells, and sperm cells. Some macrophages were mannose receptor-negative: the microglial cells in the brain and the macrophages in the thymus cortex, B cell areas in lymph nodes and spleen. In tissues affected by tuberculosis, leprosy, carcinoma or rheumatoid arthritis the macrophage mannose receptor appeared to be downregulated which indicated that the macrophages were activated, whereas in a reaction against polyethylene the macrophage mannose receptor appeared to be upregulated, which indicated that the macrophages were alternatively activated. It was concluded that *in vivo* few human cell types express the mannose receptor, and that the mannose receptor expression *in vivo* also reflects the type of macrophage activation. mAb 15-2 proved to be a specific tool in studying the mannose receptor expression in human tissues.

Inhibitors of the mannose receptor-mediated t-PA clearance

A series of mannose receptor inhibitors was synthesized. The series consisted of lysine- or polylysine-based oligomannosides containing two (M_2L_1) to six (M_6L_5) terminal α-D-mannose groups that were connected to the backbone by flexible elongated spacers. The efficacy of these cluster mannosides to inhibit the interaction of t-PA or ribonuclease B with the mannose receptor was tested in the microtitre plate binding assay (chapter 6). The cluster mannosides all inhibited the binding of t-PA or ribonuclease B completely. The affinity of these inhibitors increased with the number of terminal mannose residues connected to the lysine backbone (M_2L_1 micromolar range and M_6L_5 nanomolar range). The shape of the inhibition curves indicated that the mannosides bound to multiple carbohydrate recognition domains on the mannose receptor. The nanomolar affinity of M_6L_5 makes it the most potent synthetic mannose receptor ligand yet developed, and besides the inhibition of ligand binding to the mannose receptor it also is a promising targeting device to accomplish cell-specific delivery of drugs (for instance in gene therapy) to cells that express the mannose receptor.

In chapter 7 we studied whether these cluster mannosides would also be effective t-PA clearance inhibitors in man. By use of immunohistochemistry we showed that, as in the rat liver, the human liver endothelial cells and human Kupffer cells do express the mannose receptor. The mannosides inhibited mannose receptor-mediated t-PA binding, association and degradation by isolated rat liver endothelial cells and t-PA association and degradation by cultured human macrophages with similar affinities. M_5L_4 inhibited t-PA degradation in the micromolar range whereas M_6L_5 was able to inhibit t-PA degradation in the nanomolar range. The concentrations of mannoside necessary to inhibit t-PA degradation *in vitro* were comparable to the concentrations necessary to inhibit mannose receptor-mediated t-PA clearance *in vivo* in the rat. Since there was no species difference between rat and man with respect to the distribution of the mannose receptor in the liver and the affinity of the cluster mannosides, it is likely that M_6L_5, as observed in the rat, will be an efficient inhibitor of mannose receptor-mediated t-PA clearance in man.

In chapter 8 we provide evidence for a possible antithrombotic effect of mannose receptor inhibitors *in vivo* in man. Dextran is a blood substitute with antithrombotic properties. Recently it has been shown that dextran increases the t-PA plasma concentration and activity in patients. Dextran is a glucose polymer and a potential ligand for the mannose receptor.

We studied the effect of dextran on the mannose receptor-t-PA interaction in our *in vitro* models, and *in vivo* in the rat. At therapeutic concentrations, dextran was able to inhibit t-PA binding to the isolated receptor and the mannose receptor-mediated t-PA degradation by macrophages, while leaving the LRP-mediated t-PA degradation unaffected. *In vivo* dextran increased endogenous t-PA concentrations and inhibited exogenous t-PA clearance. Furthermore, the endogenous t-PA concentrations correlated strongly with the clearance of exogenous t-PA. We concluded that the dextran-induced increase of endogenous t-PA concentrations are a result of the inhibition of mannose receptor-mediated t-PA clearance by dextran, which is a new explanation for the antithrombotic effect of dextran.

In chapter 9 the role of the mannose receptor and other clearance receptors in the regulation of t-PA concentrations is discussed. We concluded that the plasma clearance of t-PA is mainly mediated by the mannose receptor and the LRP expressed in the liver. The high mannose-type oligosaccharide of t-PA binds to the mannose receptor; the growth factor and finger domain are involved in the binding of t-PA to the LRP. The expression of these receptors in the liver possibly influences the plasma concentrations of t-PA under (patho)physiological conditions such as ageing, pregnancy, obesity and diabetes. Inhibitors of (mannose receptor-mediated) clearance might be useful in thrombolytic and antithrombotic therapy.

Conclusions and perspectives

We developed useful assays and tools to study the 175 kD human mannose receptor and high affinity ligands that are able to inhibit its interaction with t-PA. We showed that the mannose receptor is expressed on macrophages in human tissues, and that its expression depends on the type of macrophage activation. The monoclonal antibodies against the mannose receptor may offer new insights into the role of macrophages in various diseases. The mannose receptor expressed on liver endothelial cells and Kupffer cells plays an important role in the clearance of t-PA. It was demonstrated that mannose receptor ligands could partially inhibit the clearance of exogenous t-PA and increase endogenous t-PA plasma concentrations. Thus mannose receptor ligands such as M_6L_5 can be considered as a new strategy to increase the t-PA concentration in blood and thereby increase the efficacy of thrombolytic and antithrombotic therapy.

The mannose receptor ligands described in this thesis may in the future besides in the treatment of thrombotic diseases also be used in the treatment of other diseases. One can think of connecting drugs to these ligands in order to target the drugs towards mannose receptor-expressing cells (sinusoidal cells in liver and spleen, and macrophages and dendritic cells in all tissues). In combination with a dextran 70 (macrodex) infusion it may be possible to target the ligand-connected drug mainly to the cells residing outside the bloodstream, since dextran inhibits the mannose receptor-mediated clearance and is too large to escape from the bloodstream. In this way drugs may be targetted towards macrophages and dendritic cells. This would make it possible to activate or suppress the immune system. One could, for example, load dendritic cells with cell type-specific antigens in order to activate the immune system to kill these cell types (such as cancer cells or cells that produce autoantibodies). It is a hopeful thought that maybe in the future we will be able to manipulate the immune system in this way.

SAMENVATTING

De mannose receptor, localisatie en rol in de klaring van weefseltype plasminogeenactivator.

Na beschadiging van een bloedvat wordt een bloedstolsel gevormd en na herstel van het weefsel wordt het stolsel weer afgebroken. Stolselvorming en stolselafbraak worden gecontroleerd door de productie en afbraak van enzymen en enzymremmers. De balans tussen deze processen bepaalt of het stolsel gevormd dan wel afgebroken wordt. Weefseltype plasminogeenactivator (t-PA) is een enzym dat plasminogeen om kan zetten in plasmine, welke de onoplosbare struktuur van een stolsel, fibrine, om kan zetten in oplosbare fibrine afbraak producten. Het t-PA dat in bloed circuleert wordt in het lichaam continu aangemaakt door de endotheelcellen die de bloedvaten bekleden, en continu opgenomen en afgebroken (klaring) door de lever. De klaring van t-PA door de lever is een zeer efficient proces; binnen 5 minuten is de helft van een toegediende dosis t-PA uit de bloedbaan verdwenen. De klaring van t-PA door de lever is een receptor-gemedieerd proces. t-PA bindt aan receptoren die op het oppervlak van de cellen in de lever zitten. Na binding wordt het receptor-t-PA complex opgenomen door de cel, het t-PA wordt afgebroken en de receptor keert terug naar het celoppervlak waar het opnieuw t-PA kan binden. Vooral de mannose receptor en de α_2-macroglobulin receptor (ook wel de "low density lipoprotein receptor-related protein" (LRP) genoemd) zijn verantwoordelijk voor de klaring van t-PA door de lever. Wanneer een bloedstolsel gevormd wordt, wordt t-PA in het stolsel ingebouwd. Hoe meer t-PA in het stolsel zit, hoe sneller het stolsel daarna afgebroken kan worden. Een grote hoeveelheid t-PA kan ervoor zorgen dat de balans van stolselvorming en stolselafbraak doorslaat naar stolselafbraak. Daarom wordt recombinant t-PA succesvol gebruikt als een thrombolytisch medicijn na bijvoorbeeld een hartinfarct, en zou infusie van lage hoeveelheiden t-PA bruikbaar kunnen zijn in antithrombotische therapie.

In dit proefschrift is de hypothese gesteld dat remmers van de mannose receptor-t-PA binding, de klaring van t-PA kunnen remmen. Door remming van de klaring zou de effectiviteit van toegediend of endogeen t-PA verhoogd kunnen worden. Deze remmers zouden dan nuttige medicamenten kunnen zijn in thrombolytische en antithrombotische therapie. Het doel van deze studie was de kennis over de werking en de functie van de mannose receptor te vergroten teneinde effectieve mannose receptor remmers te ontwikkelen. Deze kunnen in de toekomst mogelijk gebruikt worden in de behandeling van thrombotische (en andere) ziektes.

Opzet van het onderzoek

Om de humane mannose receptor-t-PA interactie te kunnen karakteriseren en de effectiviteit van remmers te kunnen evalueren ontwikkelden we twee *in vitro* meetsystemen (hoofdstuk 1 en 2). Monoclonale antilichamen (mAb) tegen de humane mannose receptor werden geproduceerd om specifiek die mannose receptor te kunnen bestuderen die in staat is om t-PA te binden (hoofdstuk 3). Om de mogelijke functie(s) van de mannose receptor in de mens vast te kunnen stellen, werden deze mAb gebruikt om de expressie van de mannose receptor op cellen *in vitro* en in gezond en ziek humaan weefsel zichtbaar te maken (hoofdstuk 4 en 5). Van een serie synthetische mannose receptor liganden en van het antithrombotische middel dextran, werd de effectiviteit om de mannose receptor-t-PA interactie in bovengenoemde *in vitro* modellen en *in vivo* te remmen, getest (hoofdstuk 6, 7 en 8). In het laatste hoofdstuk (hoofdstuk 9) worden de resultaten van deze studie bediscussieerd en wordt een overzicht gegeven van de regulatie van plasma t-PA concentraties door receptor-gemedieerde klaring.

In vitro meetmodellen van de humane mannose receptor-t-PA interactie

De mannose receptor heeft een aantal suiker herkenningsdomeinen. Hoe meer suiker herkenningsdomeinen van de mannose receptor het ligand binden, hoe hoger de affiniteit van het ligand. Uit voorgaand onderzoek was gebleken dat het hoog mannose-type suiker (een suiker polymeer met 3 eindstandige mannoseresiduen) van t-PA aan de mannose receptor bindt. In hoofdstuk 1 werd de binding van t-PA aan de geisoleerde humane mannose receptor nader gekarakteriseerd. De mannose receptor werd geisoleerd uit humane placenta, en de binding van t-PA en t-PA varianten werd gemeten in een microtiterplaat bindingsassay. Het bleek dat t-PA met een veel hogere (500 maal) affiniteit aan de mannose receptor bindt dan twee andere hoog mannose-type suiker bevattende eiwitten, ribonuclease B en ovalbumine. De binding van t-PA was specifiek aangezien het volledig geblokkeerd kon worden met mannan (een mannose polymeer) of met polyclonale antilichamen tegen de mannose receptor. Het is onwaarschijnlijk dat eiwit-eiwit interacties significant bijdragen aan de hoge affiniteits binding van t-PA aan de mannose receptor aangezien een suikerloze t-PA variant de binding van t-PA niet kon remmen. Complex vorming van t-PA met plasminogeen activator remmer type 1 (PAI-1) of α_2-antiplasmine had geen invloed op de affiniteit van t-PA voor de mannose receptor. Verder bleek dat de complex-type suikers of de fucose suiker van t-PA niet betrokken waren bij de hoge affiniteit van t-PA.

Het eiwit gedeelte van t-PA, ribonuclease B en ovalbumine werd volledig geknipt met trypsine, een enzym dat de suiker polymeren van de eiwitten intact laat. Het trypsine afbraakproduct van t-PA had een 500-voud lagere affiniteit dan intact t-PA, terwijl de trypsine afbraakproducten van ribonuclease B en ovalbumine slechts een 2- tot 3-voudige lagere affiniteit dan de intacte eiwitten hadden. Dit gaf aan dat het hoog mannose-type suiker van t-PA niet anders aan de mannose receptor bond dan de hoog mannose-type suikers van ribonuclease B en ovalbumine. Waarschijnlijk beinvloedt het eiwit van t-PA de conformatie van het hoog mannose-type suiker op t-PA zodanig dat de het suiker polymeer aan meerdere suiker herkenningsdomeinen van de mannose receptor kan binden waardoor intact t-PA een hoge affiniteit heeft voor de mannose receptor. De bindingsassay met geisoleerde humane mannose receptor werd in verdere studies als een *in vitro* meetsysteem gebruikt om de effectiviteit van remmers van de mannose receptor-t-PA binding te bepalen (zie hoofdstuk 3,6,7,8).

In hoofdstuk 2 werden de receptoren die verantwoordelijk zijn voor de binding, opname en afbraak van t-PA door gekweekte humane macrofagen gekarakteriseerd. Uit buffy coats van donor bloed, werden monocyten geïsoleerd en gekweekt. Binnen 2 dagen waren de monocyten gedifferentieerd tot macrofagen. t-PA bond bij 4°C met hoge (Kd = 1-5 nM) en lage (Kd >350 nM) affiniteit aan de macrofagen. De lage affiniteits bindingsplaatsen waren rembaar met de lysine analoog 6-aminohexaan zuur (6-AHA). Bij 37°C werd t-PA opgenomen en afgebroken door de macrofagen Dit proces was gedeeltelijk rembaar met mannan of een monoclonaal antilichaam tegen de mannose receptor (zie hoofdstuk 3). Het niet mannan-rembare deel van de afbraak kon geblokkeerd worden met receptor associated protein (RAP), een remmer van de LRP. Inactivatie van t-PA noch toevoeging van 6-AHA had invloed op de afbraak van t-PA. De afbraak van t-PA door monocyten was niet rembaar met mannan. De mannose receptor- en LRP-gemedieerde t-PA afbraak werd hoger tijdens differentiatie van monocyt tot macrofaag. Wanneer de macrofagen gestimuleerd werden door dexamethason werd alleen de mannose receptor-gemedieerde afbraak verhoogd. Wanneer de macrofagen gestimuleerd werden met lipopolysaccharide werd de mannose receptor- en de LRP-gemedieerde t-PA afbraak verlaagd. We concludeerden dat humane macrofagen twee onafhankelijk gereguleerde receptoren, de mannose receptor en de LRP, gebruiken voor de opname en afbraak van t-PA. Gekweekte humane macrofagen zijn, aangezien vooral deze twee receptoren verantwoordelijk zijn voor de klaring van t-PA in de lever, een praktisch *in vitro* meetsysteem voor de evaluatie van t-PA klaringsremmers. In verdere studies werden deze macrofagen dan ook gebruikt om de effectiviteit van mannose receptor-gemedieerde t-PA afbraak remmers te bepalen (zie hoofdstuk 3,7,8).

Ontwikkeling en toepassingen van monoclonal antilichamen tegen de humane mannose receptor
Een set van 5 monoclonale antilichamen (mAb) werd opgewekt tegen de mannose receptor geisoleerd uit humane placenta en gekarakteriseerd (hoofdstuk 3). Uit Western blots bleek dat alle mAb de 175 kD mannose receptor in het ruwe placenta extract herkenden. Met behulp van immunohistochemie toonden we aan dat de mannose receptor in de placenta tot expressie gebracht wordt door de Hofbauer cellen (foetale macrofagen). Uit remmings experimenten bleek dat de mAb minstens twee verschillende epitopen op de geisoleerde mannose receptor herkenden. Een van deze epitopen ligt in de buurt van de bindingsplaats van t-PA, aangezien de mAb die dit epitoop herkenden, de binding van t-PA aan de geisoleerd mannose receptor en de mannose receptor-gemedieerde t-PA afbraak door macrofagen sterk konden remmen.

Deze mAb werden gebruikt om de mannose receptor expressie op geïsoleerde cellen en in lymfoide organen te bestuderen (hoofdstuk 4). Met behulp van flow cytometrie bleek dat geisoleerde humane monocyten en lymfocyten niet en gekweekte macrofagen wel de mannose receptor tot expressie brengen. Dexamethason behandelde macrofagen brachten meer mannose receptor tot expressie en lipopolysaccharide behandelde macrofagen brachten minder mannose receptor tot expressie. De mannose receptor expressie van een aantal celtypes die betrokken zijn bij de afweer werd bestudeerd met behulp van immunohistochemie (met mAb 15-2). Monocyten, lymfocyten, granulocyten en Langerhans cellen waren mannose receptor-negatief, weefsel macrofagen in thymus, lymfknopen, beenmerg en huid waren mannose receptor-positief. Opvallend was dat de macrofagen in thymus schors en de T cel gebieden van de lymfknopen mannose receptor-positief waren, terwijl de macrofagen in de thymus merg en de B cel gebieden van de lymfknopen mannose receptor-negatief waren.

Monocyten kunnen zich differentieren tot antigen presenterende cellen (dendritische cellen). Bekend is dat de mannose receptor een rol speelt bij de opname van antigenen door dendritische cellen *in vitro*. Als deze cellen gestimuleerd worden om antigen te presenteren aan T cellen, dan is de mannose receptor expressie verlaagd. Antigen presentatie vindt plaats in de thymus schors en in de B cel gebieden van lymfknopen en dit zou de afwezigheid van mannose receptor-positieve cellen in deze gebieden kunnen verklaren.

De mannose receptor is behalve bij de klaring van hoog mannose-type suikers bevattende eiwitten in de lever, ook betrokken bij andere processen. Uit *in vitro* experimenten is gebleken dat de mannose receptor betrokken is bij de complement onafhankelijke opname van pathogenen zoals Candida en Tuberculose, bij een afweerreactie tegen lichaamsvreemde stoffen waarbij macrofagen de mannose receptor waarschijnlijk gebruiken om te fuseren tot reuze cellen, bij de 'homing' van lymfocyten naar de milt, en bij de vruchtbaarheid van spermacellen. De mannose receptor expressie op macrofagen correleert met het type van macrofaag activatie *in vitro*; het T-helper type 1 cytokine γ-interferon activeert de macrofagen op zo'n manier dat de mannose receptor expressie wordt verlaagd, terwijl de T-helper type 2 cytokines interleukine-4 en interleukine-13 de macrofagen op zo'n manier activeren dat de mannose receptor wordt verhoogd (ook wel immunosuppressie of alternatieve activatie genoemd).
Met behulp van één van de mAb (mAb 15-2) hebben we in hoofdstuk 5 de weefsel distributie van de mannose receptor in gezond en ziek menselijk weefsel geevalueerd. Slechts een klein aantal celtypes brengt de mannose receptor tot expressie in gezond weefsel; de residente macrofagen in alle weefsels, de sinusoidale endotheel cellen in de lever en milt, en de spermacellen. Sommige macrofagen waren mannose receptor-negatief: de microgliacellen in de hersenen en de macrofagen in de thymus schors en in de B cel gebieden van de milt en lymfknopen. In tuberculose, lepra, kanker of reumatoide arthritis aangetaste weefsels leek de mannose receptor expressie op macrofagen verlaagd te zijn wat aangeeft dat de macrofagen geactiveerd zijn, terwijl in een afweereactie tegen polyethyleen de mannose receptor expressie verhoogd leek te zijn wat aangeeft dat de macrofagen alternatief geactiveerd zijn. We hebben geconcludeerd uit deze studie dat de mannose receptor *in vivo* slechts door een aantal cellen tot expressie wordt gebracht, en dat de mannose receptor expressie ook *in vivo* correleert met het type van macrofaag activatie. mAb 15-2 bleek een specifiek middel om de mannose receptor expressie in weefsels aan te tonen.

Remmers van de mannose receptor-gemedieerde t-PA klaring
Een serie mannose receptor liganden werd gesynthetiseerd. Aan een skelet van lysine of polylysine werden, via flexibele tussenstukken, twee (M_2L_1) tot zes eindstandige α-D-mannose residuen (M_6L_5) gekoppeld. De effectiviteit van deze 'cluster mannosides' om de t-PA of de ribonuclease B binding aan de geïsoleerde mannose receptor te remmen, werd getest (hoofdstuk 6). Alle cluster mannosides remden de binding van t-PA of ribonuclease B compleet. De affiniteit van de remmers nam toe met het aantal eindstandige mannose residuen dat aan het lysineskelet was gekoppeld (M_2L_1 micromolair affiniteit, M_6L_5 nanomolair affiniteit). De vorm van de remmings curves gaf aan dat de clustermannosides aan meerdere suiker herkenningsdomeinen van de mannose receptor kunnen binden. De nanomolair affiniteit van M_6L_5 maakt het tot het meest krachtige synthetische mannose receptor ligand tot nog toe ontwikkeld, en is behalve als remmer voor ligand binding aan de mannose receptor een veelbelovend middel om medicamenten (zoals gentherapeutica) specifiek af te leveren bij celtypes die de mannose receptor tot expressie brengen.

In hoofdstuk 7 werd getest of deze cluster mannosides ook effectieve t-PA klaringsremmers in de mens zouden kunnen zijn. Allereerst toonden we met behulp van immunohistochemie aan dat, net zoals in de rattelever, in de humane lever de mannose receptor tot expressie wordt gebracht door lever sinusoidale endotheelcellen en Kupffercellen. De mannosides remden met vergelijkbare affiniteit de mannose receptor-gemedieerde t-PA binding, opname en afbraak door sinusoidale endotheelcellen geïsoleerd uit rattelevers en de t-PA opname en afbraak door gekweekte humane macrofagen. M_5L_4 remde t-PA afbraak met micromolair affiniteit terwijl M_6L_5 de t-PA afbraak met nanomolair affiniteit remde. De concentraties nodig om t-PA afbraak *in vitro* te remmen waren vergelijkbaar met de concentraties nodig om t-PA klaring *in vivo* in de rat te remmen. Het is waarschijnlijk dat M_6L_5, zoals waargenomen in de rat, een efficiente remmer van de t-PA klaring in de mens zal zijn, aangezien er geen species verschil tussen de expressie van de mannose receptor in de lever en in de affiniteit van remming was.

In hoofdstuk 8 leveren we aanwijzingen dat mannose receptor remmers *in vivo* in de mens, mogelijk een antithrombotisch effect hebben. Dextran is een bloedvervanger met een antithrombotische werking. Recente studies hebben aangetoond dat dextran de plasma t-PA concentratie en activiteit verhoogt in patienten. Dextran is een glucose polymeer en een mogelijk ligand van de mannose receptor. We hebben het effect van dextran op de mannose receptor-t-PA interactie in onze beide *in vitro* modellen en *in vivo* in de rat getest. Bij therapeutische concentraties, bleek dextran in staat om de binding van t-PA aan de geïsoleerde receptor, en de mannose receptor-gemedieerde t-PA afbraak door macrofagen te remmen, terwijl de LRP-gemedieerde t-PA afbraak onaangetast bleef. Dextran bleek *in vivo* endogene t-PA concentraties te verhogen en de klaring van exogeen t-PA te remmen. Tevens correleerde de endogene t-PA concentraties met de klaringssnelheid van exogeen t-PA. We concludeerden dat de dextran gemedieerde verhoging van endogene t-PA concentraties een gevolg is van de remming van de mannose receptor-gemedieerde t-PA klaring door dextran, hetgeen een nieuwe verklaring is voor het antithrombotische effect van dextran.

In hoofdstuk 9 werd de rol van de mannose receptor en andere klarings receptoren in de regulatie van t-PA concentraties geevalueerd. Hierin concluderen we dat de plasmaklaring van t-PA voornamelijk gemedieerd wordt door de mannose receptor en de LRP in de lever. Het hoog mannose-type suiker van t-PA bindt aan de mannose receptor, terwijl het groeifactor- en het vinger-domein van t-PA betrokken zijn bij de binding aan LRP. De expressie van deze receptoren in de lever beinvloedt mogelijk de endogene plasma t-PA concentraties bij (patho)fysiologische condities zoals veroudering, zwangerschap, overgewicht en diabetes. Remmers van de (mannose receptor-gemedieerde) t-PA klaring zijn een mogelijkheid om de effectiviteit van thrombolytische en antithrombotische therapie te verhogen.

Conclusies en toekomstperspectief

In deze studie werden nuttige meetmodellen en middelen om de 175 kDa mannose receptor te bestuderen, en hoge affiniteits liganden om zijn interactie met t-PA te remmen ontwikkeld. We toonden aan dat in menselijke weefsel de mannose receptor door macrofagen tot expressie wordt gebracht, en dat deze expressie afhankelijk is van het type van macrofaag activatie. De monoclonale antilichamen tegen de mannose receptor kunnen mogelijk nieuwe informatie verschaffen over de rol van macrofagen in verschillende ziekten. De mannose receptor op lever sinusoidale endotheelcellen en Kupffercellen speelt een belangrijke rol in de klaring van t-PA. Het bleek dat mannose receptor liganden/remmers de klaring van exogene t-PA gedeeltelijk konden remmen, en de endogene t-PA plasma concentratie konden verhogen. Mannose receptor remmers zoals M_6L_5 kunnen dus beschouwd worden als een nieuwe strategie om de t-PA concentratie in bloed te verhogen en daarmee de effectiviteit van thrombolytische en antithrombotische therapie te verhogen.

De in dit proefschrift beschreven mannose receptor liganden zouden behalve bij de behandeling van thrombotische aandoeningen in de toekomst misschien gebruikt kunnen worden bij de behandeling van andere ziektes. Men kan daarbij denken aan koppeling van medicijnen aan deze liganden om de medicijnen specifiek te sturen naar mannose receptor bevattende cellen (de sinusoidale cellen in de lever en milt, en de macrofagen en dendritische cellen in alle weefsels). Om vooral die cellen die zich buiten de bloedbaan bevinden te bereiken, zouden ligand-gekoppelde therapeutica in combinatie met dextran 70 (macrodex) gebruikt kunnen worden. Dextran 70 remt immers de mannose receptor-gemedieerde klaring en is te groot om uit de bloedbaan ontsnappen. Op deze manier zouden therapeutica naar macrofagen en dendritische cellen gestuurd kunnen worden waardoor het mogelijk wordt om het afweersysteem te activeren of te onderdrukken. Men zou dan bijvoorbeeld het afweersysteem kunnen activeren om bepaalde celtypes te doden (zoals kankercellen of afweercellen die verkeerde antilichamen aanmaken), door dendritische cellen op te laden met antigenen van die celtypes. Het is een hoopvolle gedachte dat we in de toekomst misschien op deze manier in staat zullen zijn om het afweersysteem van het lichaam te manipuleren.

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CURRICULUM VITAE

Femke Noorman werd geboren op 15 september 1966 te Hengelo. In 1984 beëindigde zij het Gymnasium Juvenaat Heilig Hart te Bergen op Zoom en ging Biologie studeren aan de Rijksuniversiteit te Utrecht. Na het behalen van de propadeuse Biologie en het afronden van de eerste subfase van deze studie, studeerde zij van 1987 tot 1990 Bio-Farmaceutische Wetenschappen (BFW) aan de Rijksuniversiteit Leiden. In het laatste jaar van deze studie heeft zij als hoofdvak (gesubsidieerd door het Platform Alternatieven voor Proefdieren) bij de afdeling Toxicologie van BFW (o.l.v. Dr. J.F. Nagelkerke en Prof. Dr. G.J. Mulder) varken- en mensen- niercelkweken opgezet om *in vitro* niertoxiciteit te kunnen meten. In augustus 1990 behaalde zij het doctoraalexamen. Vervolgens heeft zij gewerkt als chemisch analiste bij de apotheek van het St. Elisabeth Ziekenhuis te Leiderdorp en als medewerker bij de afdeling Biofarmacie van BFW (o.l.v. Dr. J. Kuiper en Prof. Dr. Th.J.C. van Berkel). Vanaf september 1991 is zij werkzaam geweest als assistent in opleiding bij de afdeling Vaat-en Bindweefsel Onderzoek van het Gaubius Laboratorium, TNO Preventie en Gezondheid, te Leiden. Onder leiding van Dr. D.C. Rijken en Prof. Dr. P. Brakman werd het promotieonderzoek (gesubsidieerd door de Nederlandse Hartstichting) uitgevoerd zoals in dit proefschrift beschreven is.

NAWOORD

Graag wil ik alle collega's bij het Gaubius Laboratorium van TNO-PG, de afdeling Biofarmacie van het Leiden/Amsterdam Center for Drug Research en de afdeling Pathologie van het Slotervaart Ziekenhuis in Amsterdam, familieleden en vrienden bedanken voor hun bijdrage aan het tot stand komen van dit proefschrift. De volgende mensen wil ik in het bijzonder bedanken: Dick, Marrie, Ellen, Erik en Ellis voor hun aandeel in het werk, Helen voor de engelse correcties, Jaap voor de goede verzorging van Noppes de geit, Ria, Monique en Jan voor gezelligheid en inspiratie, en mijn ouders en Xris voor hun niet aflatende morele steun.

APPENDIX

Coloured figures belonging to chapter 3, 5 and 7

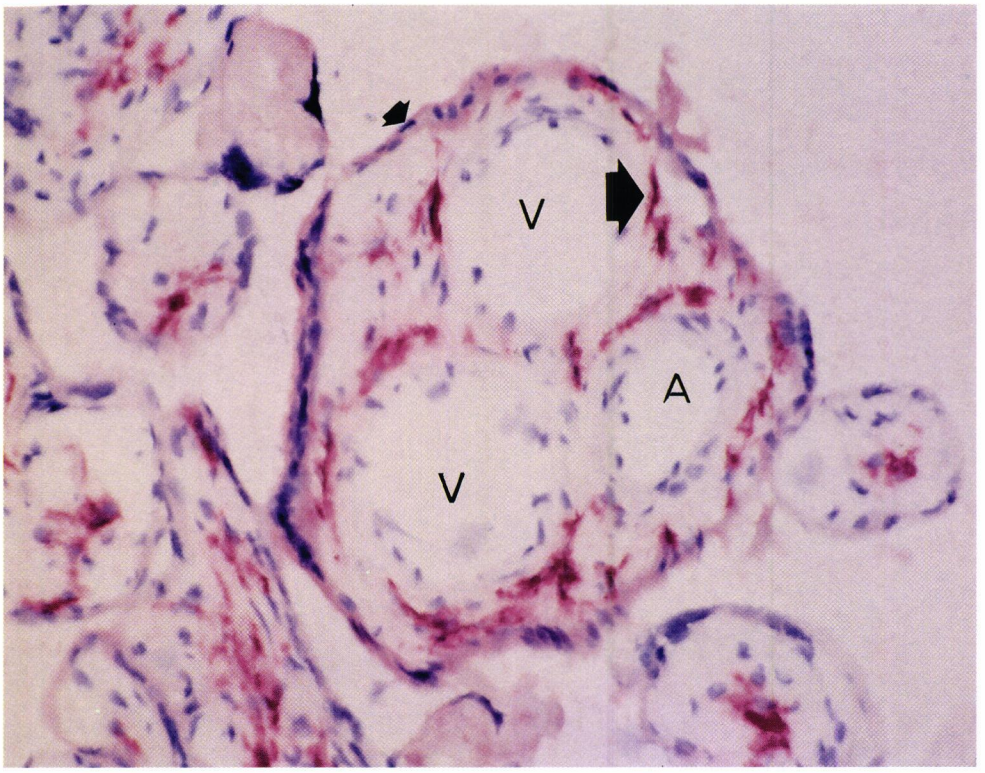


Figure belonging to chapter 3;
Monoclonal antibodies against the human mannose receptor that inhibit the binding of tissue-type plasminogen activator. ***Thrombosis and Haemostasis 1997; 77:718-724***

Fig 2 **Immunohistochemical staining of the mannose receptor in human placenta.**

Typical staining pattern (by use of mAb 15-2) of a cryostat section of human placenta. In the middle a cross section of a chorionic villus is shown. In the periphery of the villus the mannose receptor negative syncytiotrophoblasts (small arrow) are located that form the interface between maternal blood and fetal tissue. In the centre of the villus one can discriminate one artery (A) and two venules (V) surrounded by a stroma where the mannose receptor positive (intense red staining, large arrow) Hofbauer cells are located.

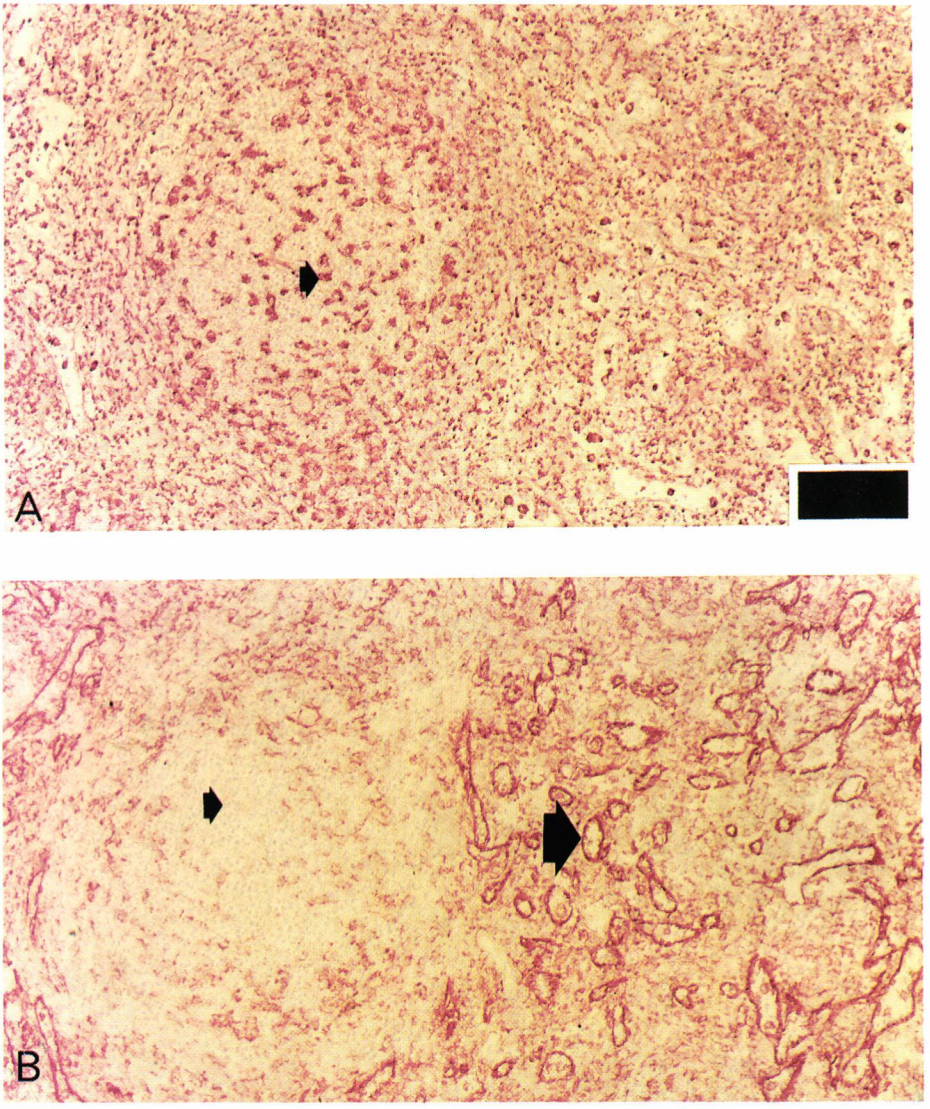


Figure belonging to chapter 5;

Expression of the human mannose receptor under physiological and pathological conditions; An immunohistochemical study using monoclonal antibody 15-2. (*submitted*)

Fig 1A-B **Mannose receptor and CD68 expression in tissue under physiological conditions.**
The distribution of CD68 (**A**) and the mannose receptor (**B**) in spleen. Bar represents 400 µm.

In the spleen, the macrophages inside the germinal centres (follicular dendritic cells, small arrow) were CD68 positive. In this area only few cells were mannose receptor-positive (small arrow). The splenic sinusoidal endothelial cells however abundantly express the mannose receptor (large arrow).

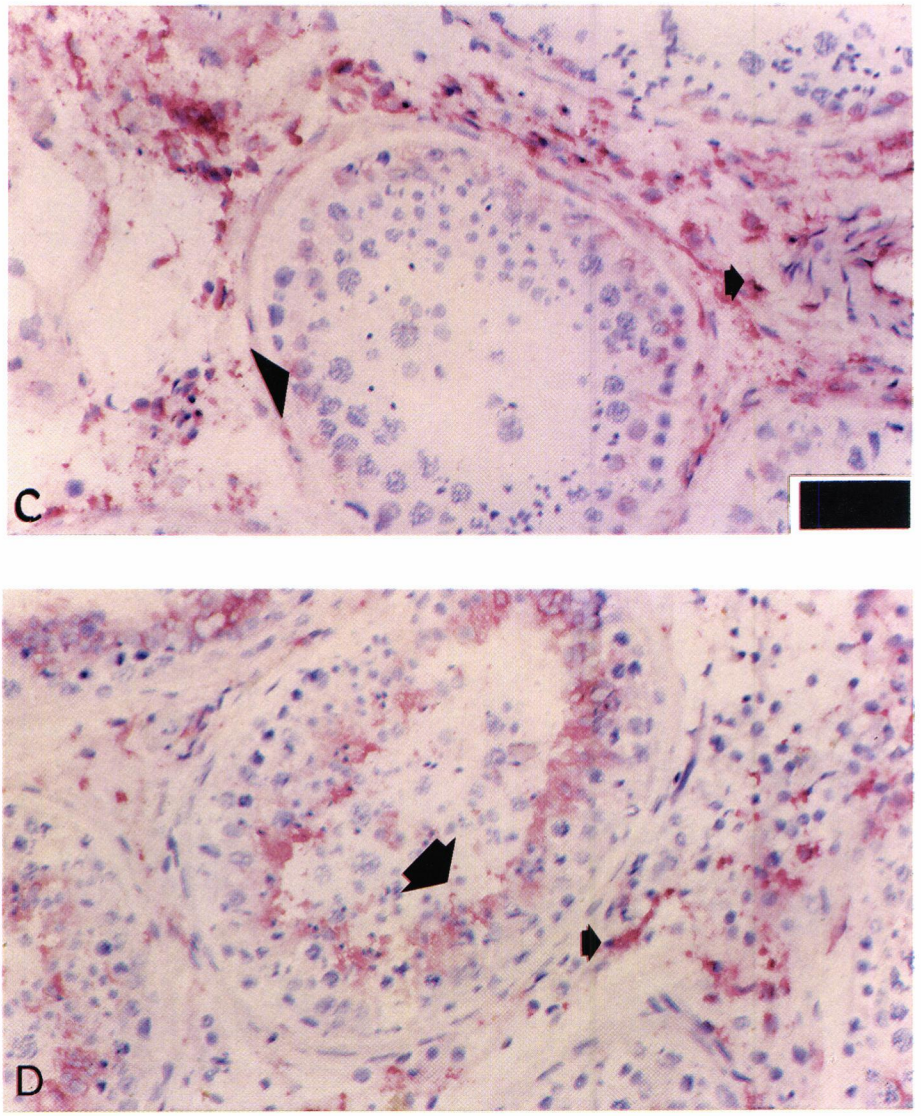


Figure belonging to chapter 5;

Expression of the human mannose receptor under physiological and pathological conditions; An immunohistochemical study using monoclonal antibody 15-2. (*submitted*)

Fig 1C-D **Mannose receptor and CD68 expression in tissue under physiological conditions.**
The distribution of CD68 (**C**) and the mannose receptor (**D**) in testis. Bar represents 200 µm.

In the testis, the resident macrophages surrounding the tubuli were mannose receptor- and CD68-positive (small arrow). The Leydig cells were CD68-positive (arrowhead) and mannose receptor-negative, whereas the spermatids and spermatozoa (large arrow) inside the seminoferous tubuli were mannose receptor-positive and CD68-negative.

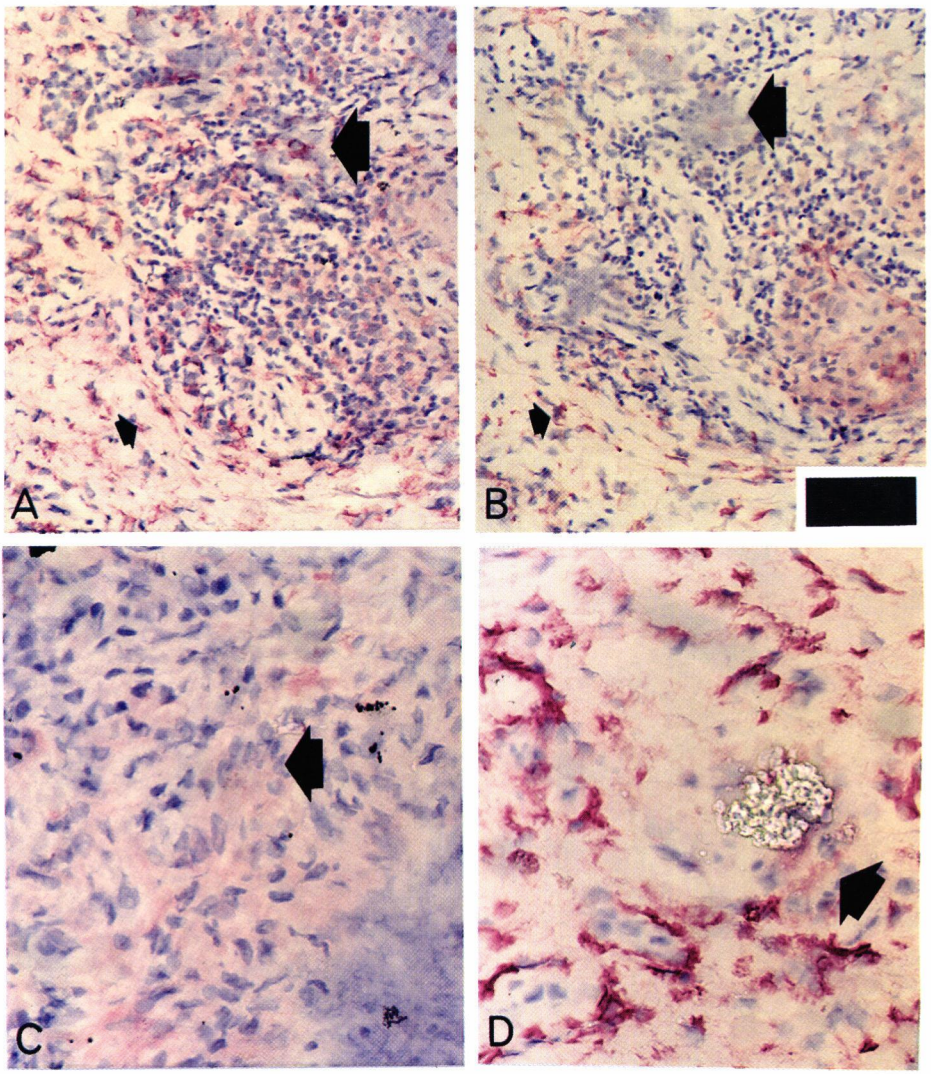


Figure belonging to chapter 5;

Expression of the human mannose receptor under physiological and pathological conditions; An immunohistochemical study using monoclonal antibody 15-2. (*submitted*)

Fig 2A-D **Mannose receptor and CD68 expression in tissue under pathological conditions.**
Tissues under various pathological conditions were stained with KP-1 (**A**) or mAb 15-2 (**B,C,D**). Bar represents 200 µm (**A,B**) or 100 µm (**C,D**).

The edge of a granuloma of skin affected by tuberculoid leprosy is shown (**A,B**). Unaffected skin tissue contained CD68-positive (**A**) and mannose receptor-positive (**B**) resident macrophages (small arrow). Inside the granulomas CD68-positive (**A**) and few weakly mannose receptor-positive macrophages (**B**) were present. The Langhans-type giant cells (large arrow) were slightly CD68-positive and mannose receptor-negative. Giant cells in tuberculosis affected lung (**C**), and in the synovium affected by a foreign body reaction (**D**) stained weakly with mAb 15-2 (large arrow). The macrophages surrounding the Langhans-type giant cells (**C**) also only weakly stained with mAb 15-2, whereas the macrophages surrounding the foreign body-type giant cells strongly stained with mAb 15-2 (**D**).

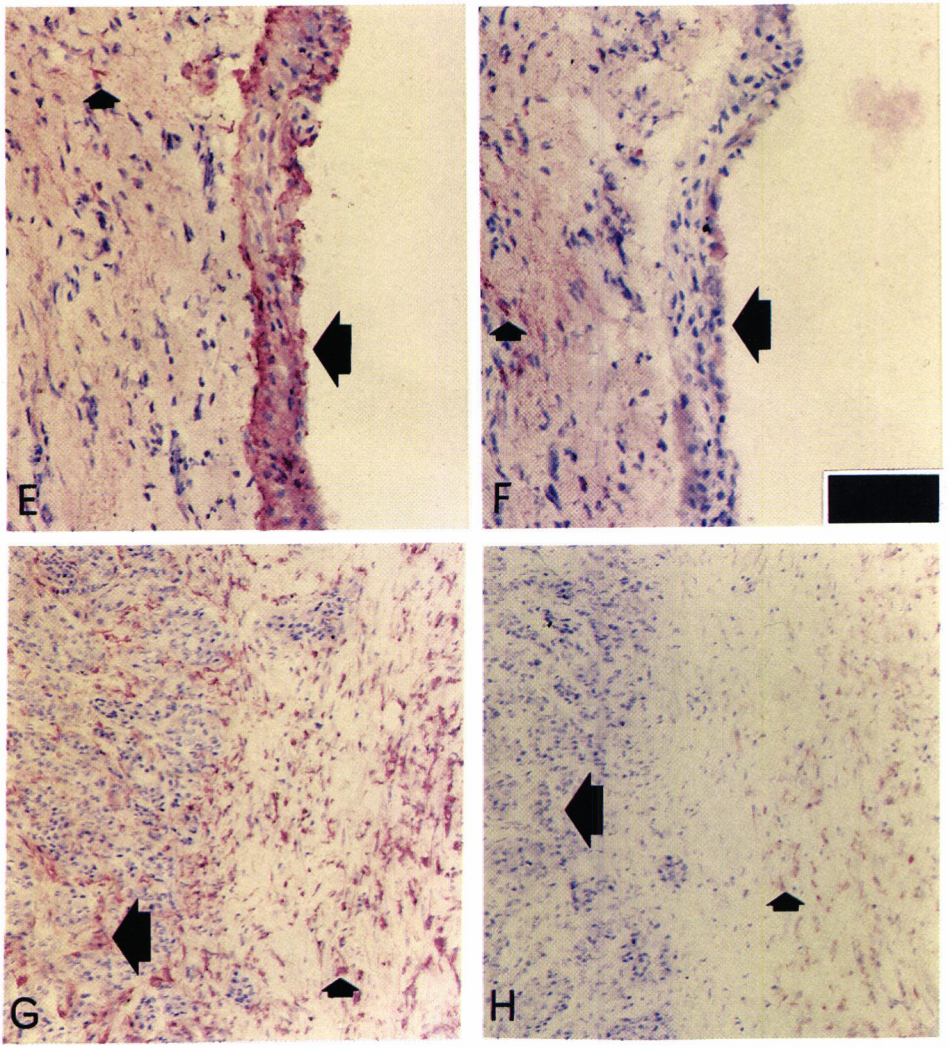


Figure belonging to chapter 5;

Expression of the human mannose receptor under physiological and pathological conditions; An immunohistochemical study using monoclonal antibody 15-2. (*submitted*)

Fig 2E-H **Mannose receptor and CD68 expression in tissue under pathological conditions.**
Tissues under various pathological conditions were stained with KP-1 (**E,G**) or mAb 15-2 (**F,H**). Bar represents 200 µm (**E,F**) or 400 µm (**G,H**).

In a synovium affected by rheumatoid arthritis most of the hypertrophic and hyperplastic synovial lining cells (large arrow) were CD68-positive (**E**) and mannose receptor-negative (**F**), whereas the stromal macrophages were CD68- and mannose receptor-positive (small arrow). In breast affected by cancer (ductal type) normal stroma macrophages (small arrow) still expressed the CD68 antigen (**G**) and the mannose receptor (**H**). Inside the desmoplastic tumor stroma the macrophages were mannose receptor-negative and CD68-positive (large arrow).

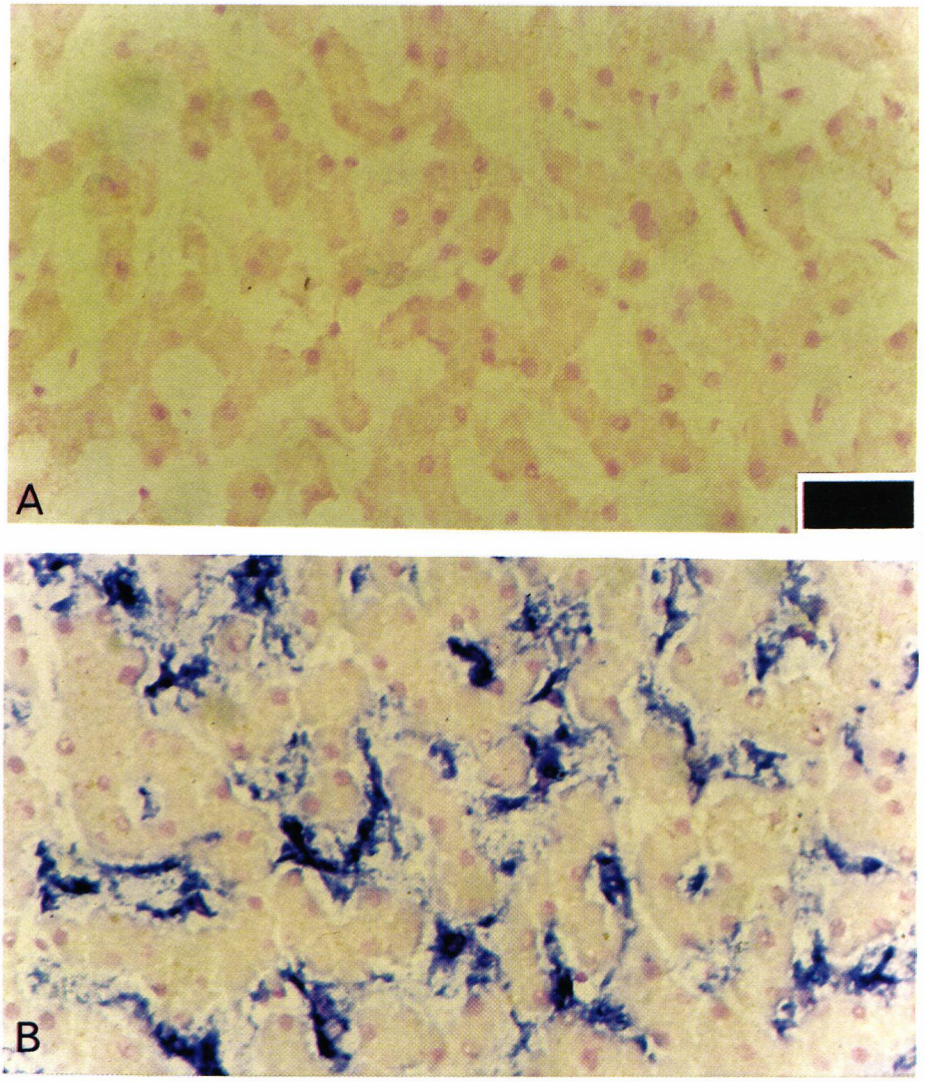


Figure belonging to chapter 7;

Cluster mannosides can inhibit mannose receptor-mediated tissue-type plasminogen activator (t-PA) degradation by both rat and human cells. *Hepatology 1997, in press.*

Fig 1A,B **The presence of mannose receptor on human liver endothelial cells and Kupffer cells.**

Representative area of a cryostat section of human liver stained without (**A**) or with (**B**) anti-mannose receptor monoclonal antibody 15-2 (blue) (both with red nuclear staining). Bar represents 100 µm (light microscope; magnification x40).

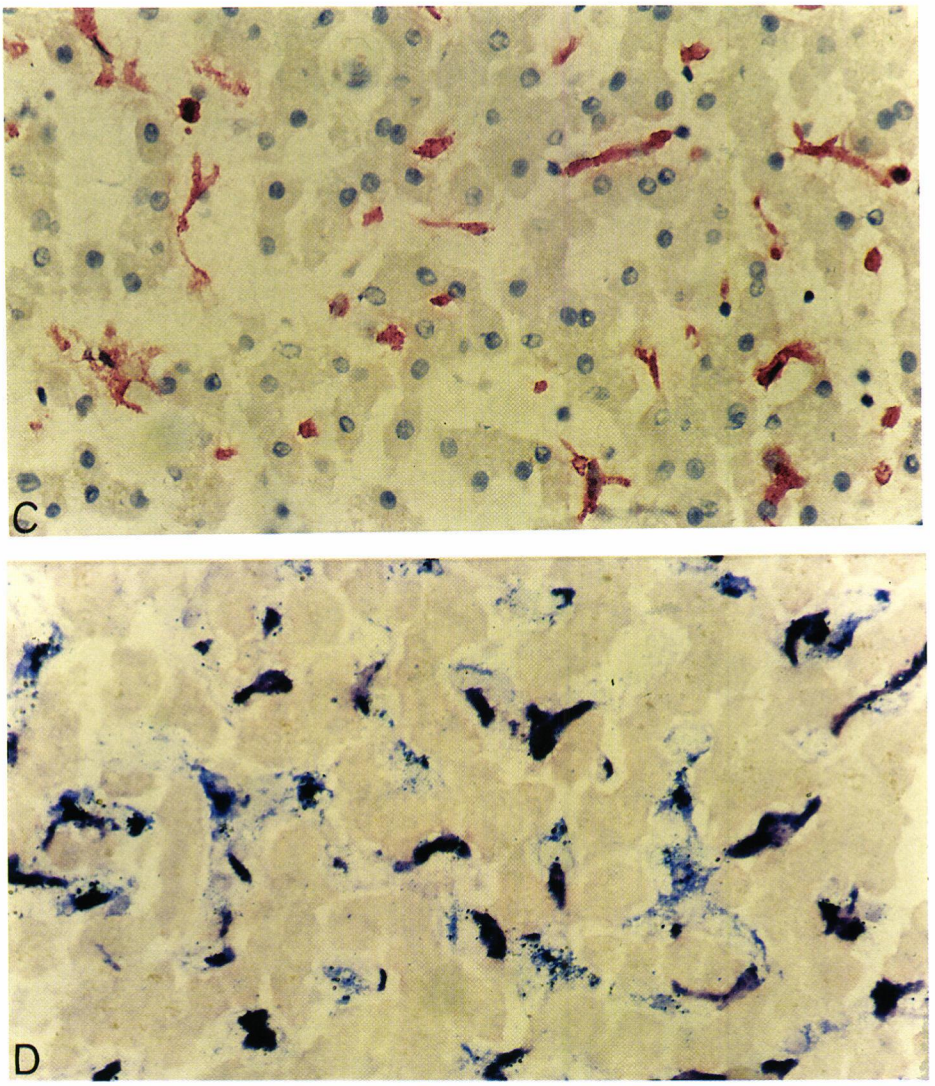
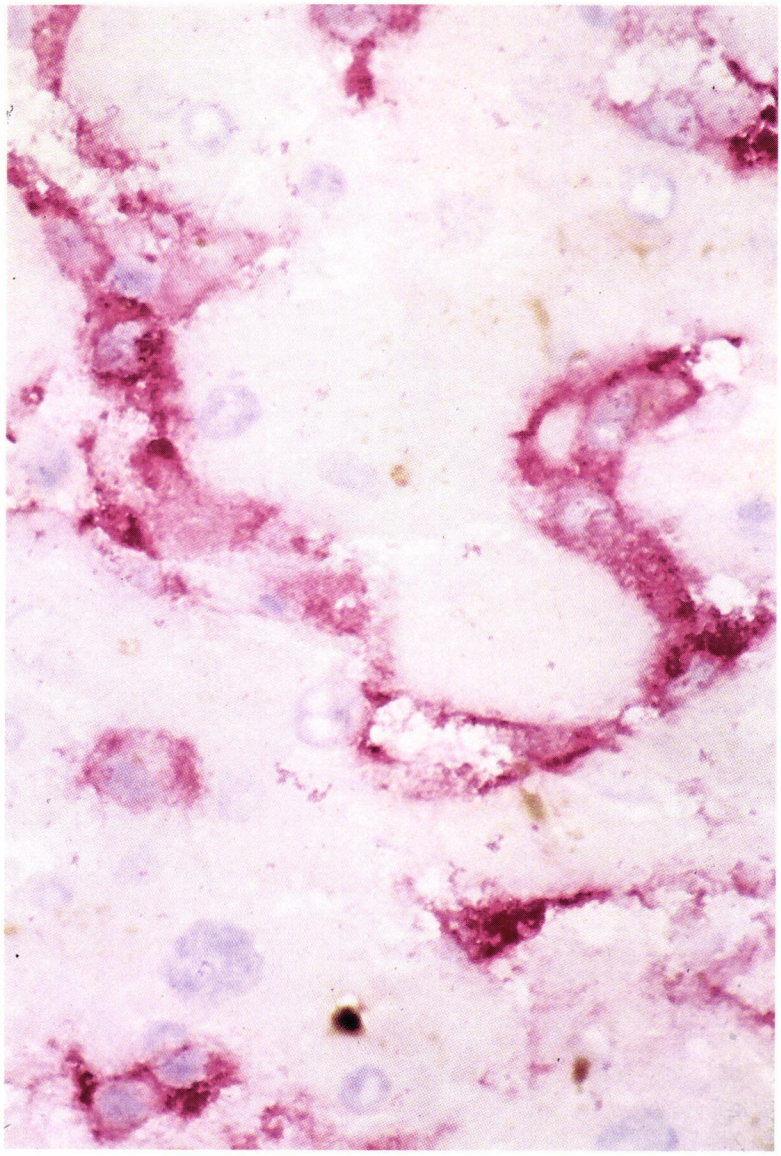


Figure belonging to chapter 7;

Cluster mannosides can inhibit mannose receptor-mediated tissue-type plasminogen activator (t-PA) degradation by both rat and human cells. *Hepatology 1997, in press.*

Fig 1C,D **The presence of mannose receptor on human liver endothelial cells and Kupffer cells.**

Representative area of a cryostat section of human liver stained with (**C**) anti CD68 monoclonal antibody KP-1 (red) (blue nuclear staining). The double staining with (**D**) KP-1 (red) plus mAb 15-2 (blue) (no nuclear staining) shows that sinus liver endothelial cells are mannose receptor-positive and CD68-negative (blue), and the Kupffer cells are both CD68- and mannose receptor-positive (purple).



Mannose receptor staining in the human liver with mAb 15-2.

This picture was used for the design of the cover of this thesis. Both Kupffer cells and liver sinusoidal cells express the mannose receptor and stain intensely red with mAb 15-2. The staining procedure was the same procedure as used for the staining of the mannose receptor in the placenta (chapter 3). Because it is difficult to discriminate between the Kupffer cells and the sinusoidal endothelial cells we performed a double staining in *Hepatology* (see the pictures on page 148,149).