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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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, [125I]-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 mann-an-inhibitable t-PA degradation. Lipopolysaccharide downregulated both mann-an-inhibitable and non-mann-an-inhibitable t-PA degradation. Non-mann-an-inhibitable degradation was completely blocked by recombinant 39-kD receptor-associated protein (RAP, inhibitor of lipoprotein receptor-related protein [LRP]), whereas mann-an-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.

MATERIALS AND METHODS

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; Boser, Organan 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 in our laboratory, and recombinant t-PA (Aclylase) 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. The 6-aminohexanoic acid (6-AHA; Merck Schuchardt, München, Germany), mannose-BSA (mannose 26 mol/mol BSA) and mannran extracted from Saccharomyces cerevisiae were used for these experiments. From the GauBius Laboratory, TNO Prevention and Health, Leiden, The Netherlands.

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charyomyces 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, provided by Dr J. Herz, University of Texas Southwestern Medical Center, Dallas, TX) were used for inhibition studies.

Cell isolation and cultivation. Buffy coat (180 mL) was diluted with 100 mL phosphate buffer (4.5 mmol/L KCL, 228 mmol/L NaCl, 13.5 mmol/L NaHPO4, 2.5 mmol/L KH2PO4, pH 7.4) containing 10 U/mL heparin. Portions of 35-mL suspension were underlayered with 14 mL Ficol-Paque or Lymphoprep. After 30 minutes’ centrifugation at 800g, the interface was collected and washed (250g, 10 minutes) twice with phosphate-buffered saline (PBS: 2.7 mmol/L KCl, 137 mmol/L NaCl, 8.1 mmol/L NaHPO4, 1.46 mmol/L KH2PO4, pH 7.4, containing 0.9 mmol/L CaCl2, 0.5 mmol/L MgCl2, and 0.1% BSA). Routinely, 1 to 2 x 10^7 cells were obtained. Cells were suspended in M199, and 5 x 10^7 cells per well were seeded in 12-well plates. Cells were cultured at 37°C in 5% CO2/95% 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 µg/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 Weinier and Shab. 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, at a flow rate of 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 10^7 cells were obtained. Cells were plated at a concentration of 1 x 10^7 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 10^7 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 TB, 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 analyzed 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 morphologic 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 125I using the iodogen method. Polystyrene vessels of 1.5 mL were coated with 10 µg iodogen. Next, 10 µg t-PA in 50 mmol/L Tris-HCl, 0.25 mmol/L 6-AHA, 1 mmol/L NaCl, pH 8.5 was incubated with 0.5 mg/mL NaI and 1 µCi 125I for 10 minutes at room temperature in the vessel. The mixture was transferred to another vessel containing an equal volume of 4 mg/mL KI to avoid nonspecific binding of 125I to t-PA. Labeled t-PA was separated from free 125I 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/mmol, with 80% recovery of t-PA activity. When indicated, 125I-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 125I-t-PA. To remove nonadherent 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. 125I-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 CaCl2 and 0.5 mmol/L MgCl2 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 125I-t-PA was corrected for plastic binding in empty parallel wells (10% to 20% of total binding).

Association and degradation of 125I-t-PA. Cells were washed (three times with PBS, 1% BSA, pH 7.4, 4°C) and incubated with 125I-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% CO2/95% air. After incubation, cell media were collected, and trichloroacetic acid (TCA) was added (final concentration, 10% wt/vol). Non-degraded 125I-t-PA was precipitated by centrifugation (10 minutes at 15,000g). To eliminate the possibility that the cells might deiodinate rather than degrade 125I-t-PA, free 125I was extracted. To the 500 µL TCA-soluble supernatant obtained, 5 µL 40% (v/v) KI and 25 µL H2O2, 30% (vol/vol) were added. After 5 minutes, the free iodine was extracted with 800 µL chloroform. The radioactivity of the remaining 125I-tyrosine (and possibly 125I-peptides) in the upper layer (5 minutes at 15,000g), representing degraded 125I-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 125I-t-PA by unlabeled t-PA or other compounds will lead to a decreased value for 125I-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 analyzed by 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 means ± 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+...
During incubation of 5 nmol/L I25I-t-PA with macrophages, 37°C to study t-PA catabolism by metabolically active cells. 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, I25I-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 differentia-
binding association degradation

Fig 3. 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 (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 center. 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. Man- nan-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, four times upregulated after day 1 (Fig 3).

Recent studies by Bu et al.20-21 suggested that t-PA can bind to LRP, which is also expressed on monocytes/macrophages. 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 by 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-
mammalian 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 nmo/L GST-RAP reduced degradation to 13%. We concluded that the mannan-inhibitable t-PA degradation by macrophages is mediated by the mannan 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.

**DISCUSSION**

Monocytes differentiated into macrophages within 2 days after plastic attachment. The morphologic 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 nmo/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,29 activated platelets,21 melanoma cells,28 monocytes,29 monocyteid cell lines,29 endothelial cells,30 and isolated human liver membrane.30 Low affinity, 6-AHA-inhibitable binding sites may play an important role for cell bound plasminogen activator activity.31-35 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 nmo/L (14 × 105 to 18 × 105 binding sites per cell). These high affinity binding sites mediated t-PA degradation (the half-maximal inhibition of degradation of 125I-t-PA by unlabeled t-PA was 3 nmo/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 macrophage receptor is 1 nmo/L,34 and to rat liver endothelial cells expressing the mannose receptor, 4 nmo/L.35 With or without PAI-1 involvement, the kd of t-PA binding to LRP ranges from 1 to 20 nmo/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 internalization of ligands and recycling receptors. Indeed, 60% to 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.43,44 Lysosomal degradation is chloroquine and NH4Cl-inhibitable,44,45,46 as observed in our model for t-PA degradation.

LRP is already present on monocytes and variably expressed on macrophages.22,27 Monocytes do not express mannose receptors.46,47 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 hours in culture.49 Previously functional mannose receptor activities have not been detected before day 3.30,50 In our cells, mannose receptor-mediated degradation was observed after 2 days. These differences in expression may be due to culture conditions.25,51-53

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.51,52 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 hours in RAW 264.7 cells53 and that mouse macrophage mannose receptors are downregulated after activation.54 The time difference in downregulation that we ob-

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**Table 1. Anti-Mannose Receptor Inhibition of 125I-t-PA (1 nmo/L) Degradation by Three-Day-Old Macrophages**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration</th>
<th>Control</th>
<th>+Anti-Mannose Receptor MoAb 10 μg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>100 ± 3</td>
<td>56 ± 6*</td>
</tr>
<tr>
<td>Mannan</td>
<td>1 mg/mL</td>
<td>49 ± 3</td>
<td>45 ± 4*</td>
</tr>
<tr>
<td>GST-RAP</td>
<td>25 nmol/L</td>
<td>60 ± 4</td>
<td>13 ± 1*</td>
</tr>
</tbody>
</table>

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

* Significantly different from control.
served is compatible with the finding that the mannose receptor is more susceptible to downregulation during activation of macrophages than the LRP.37

Mannan, ovalbumin, mannose-BSA, all known ligands for the mannose receptor,4,14,26,56 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.17,22,56 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 to 5 nmol/L for the GST-RAP protein on t-PA degradation. This correlates well with the results of Bu et al,21,29 who observed an IC50 of 3.3 nmol/L for RAP inhibition of t-PA binding to MH1C1 cells expressing LRP31 and a kd of 5 nmol/L for RAP binding to the LRP on HEP G2 cells.20 We concluded that the mannann-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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t-PA DEGRADATION BY HUMAN MACROPHAGES


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F Noorman, EA Braat and DC Rijken