Role of the scavenger receptor in the uptake of methylamine-activated α_2 -macroglobulin by rat liver

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α_2 -Macroglobulin (α_2 M) requires activation by small nucleophiles (e.g. methylamine; giving α_2 M-Me) or proteolytic enzymes (e.g. trypsin; giving α_2 M-Tr) in order to be rapidly removed from the circulation by the liver. Separation of rat liver cells into parenchymal, endothelial and Kupffer cells at 10 min after injection indicates that liver uptake of α_2 M-Me is shared between parenchymal and endothelial cells, with relative contributions of 51.3 % and 48.3 % respectively of total liver-associated radioactivity. In contrast, α_2 M-Tr is almost exclusively taken up by the parenchymal cells (90.1 % of liver-associated radioactivity). A preinjection of 5 mg of poly(inosinic acid) decreased liver uptake of α_2 M-Me to 39.9 % of the control value, while it had no effect on liver uptake of α_2 M-Tr. It appears that poly(inosinic acid) specifically reduces the uptake of α_2 M-Me *in vivo* by endothelial cells, leaving uptake by parenchymal cells unaffected. *In vitro* studies with isolated liver cells indicate that the association of α_2 M-Me with endothelial cells is 21-fold higher per mg of cell protein than with parenchymal cells. The capacity of endothelial cells to degrade α_2 M-Me appears to be 46 times higher than that of parenchymal cells. Competition studies show that poly(inosinic acid) or acetylated low-density lipoprotein effectively competes with the association of α_2 M-Me with endothelial and Kupffer cells, but association with parenchymal cells is unaffected. It is suggested that activation of α_2 M by methylamine induces a charge distribution on the protein which triggers specific uptake by the scavenger receptor on endothelial cells. It is concluded that the uptake of α_2 M-Me by the scavenger receptor might function as an additional system for the uptake of activated α_2 M.

INTRODUCTION

α_2 -Macroglobulin (α_2 M) is a 718 kDa plasma protein, composed of four identical subunits [1]. Its function is to protect the body from circulating proteolytic enzymes. In the rat, α_2 M is an acute-phase protein [2], while in man the concentration of α_2 M is relatively constant. α_2 M circulates in the blood in the 'slow' or unactivated form, with a half-life of several hours [3,4]. The reaction of α_2 M with a proteinase in the 'bait' region of the molecule [1,5] results in a conformational change which exposes previously concealed receptor-binding sequences. This form of the molecule is referred to as the active or 'fast' form (as determined by non-denaturing PAGE), and shows a half-life in the blood of 2–4 min [3,4,6]. Activation of α_2 M by primary amines such as methylamine cleaves the internal thioester bond, and this results in a similar conversion to the so-called fast form [3,4,7,8]. Both activated forms are rapidly taken up by the liver [6,9]. Although activation of α_2 M by methylamine may be considered to be less physiological than activation with a proteinase (such as trypsin), many studies have used methylamine-activated α_2 M (α_2 M-Me) for both studies *in vivo* and *in vitro* [3,4,7–11], including ourselves [12]. Studies on the structural changes that occur in α_2 M after activation have provided evidence that the conformational change after methylamine activation is indistinguishable from that occurring on activation with proteinases [4,8,13–18]. Evidence comes from studies involving non-denaturing PAGE [4,8,13], circular dichroism [14,15], sedimentation velocity [14,15], ultraviolet absorption [14,15], intrinsic protein fluorescence [16], change in surface structure [17] and kinetics of receptor-mediated endocytosis by macrophages and fibroblasts [4,7,18].

Electron-microscopic studies showed that the uptake of

trypsin-activated α_2 M (α_2 M-Tr) *in vivo* was carried out by the reticuloendothelial system in dogs [19], while liver parenchymal cells were reported to be important for uptake of α_2 M-Tr in rats [6] and of α_2 M-Me in mice [9]. Isolation of the different liver cells after injection of radiolabelled α_2 M-Me indicated that liver uptake was shared equally between parenchymal and endothelial cells [12]. *In vitro* studies showed that the uptake of α_2 M-Tr occurs by receptor-mediated endocytosis in rat parenchymal cells [20,21], rat adipocytes [20], human fibroblasts [22] and mouse and rabbit macrophages [3,18,23]. Binding of α_2 M-Tr to rat and human liver membranes and rabbit alveolar macrophages was shown to be pH- and Ca^{2+} -dependent [24–26]. Moestrup & Gliemann [27] recently analysed the binding of α_2 M-Tr to human liver membranes and proposed a model in which binding is composed of high- and low-affinity components (K_d 40 pM and 2 nM respectively, both at 4 °C). During our liver cell uptake studies we became aware that α_2 M-Tr and α_2 M-Me may trigger different receptor systems. The subject of this paper is the characterization of the liver uptake sites for α_2 M-Tr and α_2 M-Me.

MATERIALS AND METHODS

Chemicals

Collagenase, Pronase, trypsin and soybean trypsin inhibitor were from Boehringer, Mannheim, Germany. Benzamidine, phenylmethanesulphonyl fluoride and polyethylene were from Merck, Darmstadt, Germany. BSA (fraction V), poly(inosinic acid) [poly(I)] 3,3'-diaminobenzidine and agarose were from Sigma, St. Louis, MO, U.S.A. ^{125}I (carrier free) in NaOH was from Amersham. DEAE-Sepharose Fast Flow, Sephacryl S-300 Superfine, metal-chelating Sepharose Fast Flow and Mono Q

Abbreviations used: LDL, low-density lipoprotein; Ac-LDL, acetylated LDL; α_2 M, α_2 -macroglobulin; α_2 M-Me, methylamine-activated α_2 M; α_2 M-Tr, trypsin-activated α_2 M; poly(I), poly(inosinic acid); DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline.

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were from Pharmacia, Uppsala, Sweden. Bio-Gel A-1.5 m was from Bio-Rad. All other chemicals were of analytical grade.

α_2 M and low-density lipoprotein (LDL)

Human and rat α_2 M were isolated from fresh EDTA-treated plasma essentially according to the method of Sottrup-Jensen *et al.* [28], with slight modifications introduced by Lonberg-Holm *et al.* [29], using Zn^{2+} chelate affinity chromatography. Benzamidine and phenylmethanesulphonyl fluoride were added to plasma up to final concentrations of 5 mM and 2 mM respectively, to inactivate proteolytic activity. Programmable f.p.l.c. (Pharmacia) and column media for performance at high flow rates were used. α_2 M (unlabelled or ^{125}I -labelled) was activated with either trypsin or methylamine. Activation with trypsin was achieved by incubating α_2 M with a 15-fold molar excess of trypsin for 5 min at 20 °C, followed by addition of a 5-fold molar excess of soybean trypsin inhibitor over trypsin [6]. In a subsequent step, α_2 M was purified by gel filtration on a 0.7 cm $\times$ 25 cm Bio-Gel A-1.5m column at 4 °C with 0.15 M-NaCl/8 mM-phosphate buffer (PBS) containing 1 mM-EDTA (PBS/EDTA), pH 7.4, as the eluent. Activation of α_2 M with methylamine was achieved by incubation with 0.2 M-methylamine (final concentration) for 2 h at 20 °C and subsequent dialysis against PBS/EDTA, pH 7.4, at 4 °C for 24 h, with repeated changes of buffer.

Human LDL ($1.019 < d < 1.063$) was isolated from human serum from fasted individuals by two repetitive centrifugations according to the procedure of Redgrave *et al.* [30]. Human LDL was acetylated as described before [31]. LDL (1–3 mg) in PBS/EDTA, pH 7.4, was added to a saturated solution of sodium acetate with continuous stirring in an ice/water bath. Acetic anhydride was added in small portions (2 μl) over a period of 1 h. After the addition of a total mass of acetic anhydride equal to 1.5 times the mass of protein added, the mixture was stirred for another 30 min. Finally, the solution was dialysed for 24 h against PBS/EDTA, pH 7.4, at 4 °C, with repeated changes of buffer.

Labelling of α_2 M

Unactivated α_2 M was radioiodinated with chloramine-T as described previously [6]. In brief, 350 μg of α_2 M in 200 μl of PBS, pH 8.0, was mixed with 8 μl of ^{125}I in 0.1 M-NaOH followed by addition of 214 μg of chloramine-T in 107 μl of PBS, pH 8.0. After 90 s the reaction was stopped by addition of 164 μg of $\text{Na}_2\text{S}_2\text{O}_5$ in 82 μl of PBS, pH 8.0, and 120 μg of KI in 60 μl of PBS, pH 8.0. In some experiments, ^{125}I - α_2 M was directly activated with trypsin as described above. In both cases, ^{125}I - α_2 M was purified by gel filtration on a Bio-Gel A-1.5m column as described above. The specific radioactivity of ^{125}I - α_2 M ranged from 380 to 900 c.p.m./ng of protein. The labelled products were stored at –20 °C for no more than 14 days. In some experiments, α_2 M was radioiodinated at pH 10 according to a modification [32] of the ICl method [33] or according to the solid-state lactoperoxidase method [34]. Low-molecular-mass radioactive products were removed by gel filtration on a 0.5 cm $\times$ 3 cm Sephacryl S-200 column.

Serum decay and liver uptake of α_2 M

Male Wistar rats of body weight 225–300 g, fed *ad libitum* with standard chow, were used in this study. Rats were anaesthetized by intraperitoneal injection of 15–20 mg of sodium pentobarbital and the abdomen was opened. If indicated, rats received an injection of 5 mg of poly(I) in PBS/EDTA, pH 7.4, 1 min prior to injection of radiolabelled protein. Radiolabelled protein was injected via the inferior vena cava. At the indicated times, blood samples of 0.3 ml were taken from the inferior vena cava and

allowed to clot for 30 min. The samples were centrifuged for 2 min at 16000 g and 100 μl serum samples were counted for radioactivity. The total amount of radioactivity in the serum was calculated using the equation: serum volume (ml) = $[0.0219 \times \text{body weight (g)}] + 2.66$ [35].

At the indicated times, liver lobules were excised, weighed and radioactivity was counted. The amount of liver tissue tied off at the end of the experiment did not exceed 15 % of the total liver weight. Radioactivity was corrected for that in the serum assumed to be present in the tissue at the time of sampling (85 $\mu\text{l/g}$ wet weight) [36].

Isolation of liver cells

Rats were anaesthetized and injected with the radiolabelled protein. If indicated, rats received an injection of 5 mg of poly(I) in PBS/EDTA, pH 7.4, 1 min prior to injection of radiolabelled protein. Rat liver parenchymal, endothelial and Kupffer cells were isolated by differential centrifugation and counterflow elutriation as described in detail elsewhere [37]. The contributions of the different liver cell types to the uptake of (lipo)proteins were calculated based on the assumption that 92.5 % of total liver protein is contributed by parenchymal cells, 3.3 % by endothelial cells and 2.5 % by Kupffer cells [38,39]. Kupffer and endothelial cells were more than 95 % pure, as judged by peroxidase staining [0.1 % 3,3'-diaminobenzidine in 0.05 M-Tris/HCl, 7 % sucrose, 0.1 % (v/v) 30 % H_2O_2 , pH 7.4; 20 min at 37 °C].

For *in vitro* studies, liver parenchymal, endothelial and Kupffer cells were isolated by perfusion of the liver with 0.1 % collagenase by the method of Seglen [40], modified as previously described [41]. Liver parenchymal, endothelial and Kupffer cells were purified by differential centrifugation and counterflow elutriation as described in detail elsewhere [37]. The obtained cells were resuspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 % BSA, pH 7.4.

In vitro studies with freshly isolated liver parenchymal, endothelial and Kupffer cells

For *in vitro* studies, 1×10^6 rat liver parenchymal (> 95 % viable, as judged by 0.2 % Trypan Blue exclusion), endothelial or Kupffer cells were incubated with 3.6 nM radiolabelled α_2 M and the indicated amounts of competitor in 0.5 ml of DMEM supplemented with 2 % BSA, pH 7.4. Incubations were carried out in plastic Eppendorf tubes for 10 min at 37 °C with continuous shaking. After incubation, parenchymal cells were centrifuged at 50 g for 1 min at 4 °C, and Kupffer and endothelial cells were centrifuged at 500 g for 5 min at 4 °C. The cells were washed twice with washing buffer (0.9 % NaCl, 1 mM-EDTA, 0.05 M-Tris/HCl, 5 mM- CaCl_2 , 0.2 % BSA, pH 7.4) and twice with washing buffer without BSA. Cells were lysed in 1 ml of 0.1 M-NaOH, and subsequently radioactivity and protein content were determined. Degradation of the protein was determined as follows. To 0.5 ml of the first supernatant was added 0.2 ml of 35 % trichloroacetic acid, followed by incubation at 37 °C for 30 min; the mixture was then centrifuged for 2 min at 16000 g. To 0.5 ml of the supernatant obtained after precipitation of the first supernatant with trichloroacetic acid were added 10 μl of 20 % KI and 25 μl of 30 % H_2O_2 . After 5 min at room temperature, 0.8 ml of chloroform was added and the mixture was shaken for 15 min. After centrifugation for 2 min at 16000 g the aqueous phase was counted for radioactivity. Protein contents were determined according to Lowry *et al.* [42], with BSA as an internal standard.

Electrophoretic studies

Non-denaturing PAGE was performed by using the Tris/borate system of Nelles *et al.* [43]. Radiolabelled protein

(5 μ g) was applied to a 5% polyacrylamide gel and electrophoresis was carried out at 20 mA for 2–3 h. After electrophoresis, the gel was fixed and stained with Coomassie Brilliant Blue.

For agarose gel electrophoresis, 2 μ g of 125 I-labelled α_2 M (unactivated or activated) was subjected to electrophoresis in 0.75% agarose gels at pH 8.8 in Tris/hippuric acid buffer (74 mM-Tris, 80 mM-hippuric acid, 6 mM-EDTA, 64 mM-NaOH). After electrophoresis, the gel was stained with Coomassie Brilliant Blue and cut into segments which were analysed for 125 I radioactivity.

RESULTS

Liver uptake and serum decay of α_2 M, α_2 M-Me and α_2 M-Tr

Unactivated α_2 M was cleared at a low rate from rat serum, in accordance with earlier data [4,6] (Fig. 1b). When α_2 M is treated with methylamine or proteolytic enzymes such as trypsin, it is converted into a high-affinity substrate for the α_2 M receptor [3,4,6]. The serum clearances of α_2 M-Me and α_2 M-Tr are rapid ($t_{1/2} \sim 2$ min) and almost identical (Fig. 1b). The uptake by liver of both α_2 M-Me and α_2 M-Tr is rapid, with 75.4% and 85.5% respectively of the injected dose present in this organ at 10 min after injection (Fig. 1a). At later time points the liver radioactivity decreases; with α_2 M-Tr, the liver-associated radioactivity decreases at a lower rate than for α_2 M-Me (in accordance with

Fig. 1. Liver uptake and serum decay of native α_2 M, α_2 M-Me and α_2 M-Tr in rats

Native 125 I-labelled human α_2 M (Δ), 125 I-labelled human α_2 M-Me ($\blacktriangle$) or 125 I-labelled human α_2 M-Tr ($\blacktriangledown$) was injected into anaesthetized rats. Liver association (a) and serum decay (b) were determined. Liver values are corrected for serum radioactivity. Values are means $\pm$ S.E.M. from at least three rats.

Fig. 2. Association *in vivo* of α_2 M-Me and α_2 M-Tr with parenchymal, endothelial and Kupffer cells

125 I-labelled human α_2 M-Me (a) or 125 I-labelled human α_2 M-Tr (b) were injected into anaesthetized rats. At 10 min after injection, the liver was perfused at 8 $^{\circ}$ C. After 8 min of perfusion, total liver association of radioactive proteins was determined (L), and subsequently parenchymal (PC), endothelial (EC) and Kupffer (KC) cells were isolated by a low-temperature method. Values are means $\pm$ S.E.M. from three rats.

previous data of Davidsen *et al.* [6]), possibly as a result of a less rapid degradation of α_2 M-Tr as compared with α_2 M-Me.

Injection of unactivated rat α_2 M or rat α_2 M-Me into rats resulted in similar liver uptake and serum decay *in vivo* as with their human counterparts (results not shown). Furthermore, liver uptake and serum decay *in vivo* of α_2 M-Me iodinated according to the ICl method or lactoperoxidase method were similar to that of the chloramine-T method (results not shown).

Cellular distribution of α_2 M-Me and α_2 M-Tr

We have shown earlier that the liver uptake of α_2 M-Me is equally shared between parenchymal and endothelial cells [12]. In order to analyse whether the method of activation of α_2 M influences the uptake by the various liver cell types, a low-temperature cell isolation procedure was performed, 10 min after injection of α_2 M-Me or α_2 M-Tr. After injection of α_2 M-Me, 38.2% of the injected dose was recovered in the parenchymal cells and 34.3% in the endothelial cells. Liver macrophages (Kupffer cells) were responsible for uptake of 10.0% of the injected dose (Fig. 2a). In contrast, injection of α_2 M-Tr results in a liver uptake which is almost exclusively carried out by the parenchymal cells (76.6% of the injected dose), with only a minor amount present in endothelial cells (2.4%) and Kupffer cells (4.9%) (Fig. 2b).

To analyse a possible effect of the amount of α_2 M-Me injected on the percentage uptake by the different cell types, we injected a 10-fold higher dose of α_2 M-Me (20 μ g instead of 2 μ g) into rats. Administration of the higher dose did not result in a change in

Fig. 3. Effect of poly(I) on liver uptake and serum decay of α_2 M-Me and α_2 M-Tr in rats

125 I-labelled human α_2 M-Me (Δ , $\blacktriangle$) or 125 I-labelled human α_2 M-Tr (∇ , $\blacktriangledown$) was injected into anaesthetized rats. Liver association (a, c) and serum decay (b, d) were determined with ($\blacktriangle$, $\blacktriangledown$) or without (Δ , ∇) a prior injection of 5 mg of poly(I). Liver values are corrected for serum radioactivity. Values are means $\pm$ S.E.M. from three rats.

the relative contributions of the various cell types to the liver uptake of α_2 M-Me.

When α_2 M-Me was isolated using a Bio-Gel A-1.5m column (as was done with α_2 M-Tr) instead of dialysis for 24 h, the uptake by endothelial cells was identical to that described above. Furthermore, the liver uptake pattern of rat α_2 M-Me was almost identical to that of human α_2 M-Me, with 36.2%, 30.8% and 10.9% of the injected dose recovered in parenchymal, endothelial and Kupffer cells respectively.

Effect of poly(I) on the liver uptake and serum decay of activated α_2 M

A great variety of ligands are reported to interact with endothelial cells, such as hyaluronan, denatured collagen and immune complexes (for a review see [44]). Charge modification of proteins or lipoproteins, e.g. acetylation of LDL (to give Ac-LDL), induces a rapid uptake by liver endothelial cells. This uptake is mediated for Ac-LDL by the so-called scavenger receptor (Ac-LDL receptor), and a characteristic feature is the blockade of this receptor by poly(I) [45]. To analyse whether the endothelial-cell-mediated uptake of α_2 M-Me was via the scavenger receptor, we injected α_2 M-Me after a preinjection of 5 mg of poly(I). As can be deduced from Fig. 3(a), poly(I) preinjection led to a marked decrease in liver uptake of α_2 M-Me (from 75.4% to 32.2% at 10 min after injection). As a consequence of the reduced liver uptake, the serum decay proceeded at a much lower rate, with 61.0% of the injected dose remaining in the serum at 10 min after injection (Fig. 3b). In contrast, the effect of

Fig. 4. Effect of poly(I) on the association *in vivo* of α_2 M-Me with parenchymal, endothelial and Kupffer cells

125 I-labelled human α_2 M-Me was injected into anaesthetized rats which had received a prior injection of poly(I). At 10 min after injection of the radiolabelled protein, the liver was perfused at 8 °C. After 8 min of perfusion, total liver association of radioactive protein was determined (L), and subsequently parenchymal (PC), endothelial (EC) and Kupffer (KC) cells were isolated by a low-temperature method. Values are means $\pm$ S.E.M. from two rats.

a preinjection of poly(I) on the liver uptake or serum decay of α_2 M-Tr was much less pronounced (Figs. 3c and 3d).

Isolation of the different liver cell types at 10 min after injection of α_2 M-Me following a preinjection of poly(I) showed that all of the liver-associated radioactivity was present in the parenchymal cells (Fig. 4). This indicates that poly(I) specifically blocks the association of α_2 M-Me with liver endothelial cells.

Subsequently we verified whether the fraction of the injected α_2 M-Me which is normally recognized by the endothelial cells is

Fig. 5. Ability of α_2 M-Me which is recognized by endothelial cells to interact with parenchymal cells *in vivo*

125 I-labelled human α_2 M-Me was injected into an anaesthetized rat which had received a prior injection of 5 mg of poly(I). After 30 min blood was collected by aortic puncture and the serum was isolated. A 1 ml portion of this serum was injected into a second rat which had also received a prior injection of 5 mg of poly(I). The liver uptake (▲) and serum decay (▼) of the radioiodinated protein in the second rat was determined. For comparison, the liver uptake (△) and serum decay (▽) of 125 I-labelled α_2 M-Me in a rat which had received a preinjection of poly(I) (see Figs. 3a and 3b) are given in the Figure. Liver values are corrected for serum radioactivity. Values are means $\pm$ S.E.M. of two (▲, ▼) and three (△, ▽) experiments.

still able to interact with the parenchymal cell uptake system after injection of poly(I). α_2 M-Me from a rat which received a preinjection of poly(I) was, after 30 min of circulation, re-injected into another rat which had also received a preinjection of poly(I). As Fig. 5 shows, the liver uptake under these conditions was very low (9.0 %), and at 30 min after injection the serum still contained 73.0 % of the injected dose. This indicates that the α_2 M-Me isolated from the serum of a rat preinjected with poly(I) is not readily recognized by liver parenchymal cells.

Capacity for cell association and degradation of α_2 M-Me

Earlier studies have revealed that parenchymal liver cells possess a high-affinity binding site for α_2 M-Tr [20]. To analyse the uptake system responsible for the interaction of α_2 M-Me with the various liver cell types, the cells were isolated and incubated with increasing amounts of α_2 M-Me. The time course of *in vitro* cell association of α_2 M-Me with freshly isolated endothelial and Kupffer cells was rapid, and reached a steady-state level within 10 min. Blomhoff *et al.* [46] previously reported a similar rapid association of Ac-LDL with the scavenger receptor on endothelial cells. With parenchymal cells, a steadier increase in the cell association of α_2 M-Me was observed (Fig. 6). The association of α_2 M-Me with endothelial cells as compared with parenchymal cells at 30 min of incubation was about 20-fold higher.

The time course of the appearance of trichloroacetic acid-soluble radioactivity in the water phase showed a clear lag phase of 10 min. Subsequently the degradation, especially by endothelial cells, proceeded at a relatively high rate (Fig. 6b). The amount of degradation of α_2 M-Me by parenchymal cells is rather low compared with endothelial and Kupffer cells. However, it is still significant, after a lag phase of 10 min, and reaches a value of about 20 ng/mg of cell protein at 2 h of incubation.

The capacity of parenchymal and endothelial cells to interact with α_2 M-Me is shown in Fig. 7. With rat parenchymal cells

Fig. 6. Time course of cell association and degradation of α_2 M-Me with parenchymal, endothelial and Kupffer cells

Freshly isolated rat parenchymal (●), endothelial (▲) and Kupffer (▼) cells were incubated at 37 °C with 2.6 μ g of 125 I-labelled human α_2 M-Me/ml (3.6 nM). At the indicated times the cell association (a) and degradation (b) were determined. Values are means of two experiments.

only, the cell association showed characteristics of saturation for α_2 M-Me.

Effects of pH, Mg-EGTA and Ca^{2+} on the association of α_2 M-Me with parenchymal and endothelial cells

Analysis of the pH-dependency of the cell association of α_2 M-Me indicated that the association with parenchymal cells is low at pH 6.0 and an optimum is reached at pH 7.5–9.0, in accordance with previous data [24,25]. The association with endothelial cells followed a different pattern, with a maximum at pH 7.5; at pH values both higher and lower than 7.5 a less optimal interaction was observed (Figs. 8a and 8b).

The effects of Mg-EGTA and Ca^{2+} on the association of α_2 M-Me with parenchymal and endothelial cells are shown in Fig. 9. It appears that the interaction of α_2 M-Me with parenchymal cells is almost totally dependent on the presence of Ca^{2+} . In contrast, Ca^{2+} appears not to be necessary for an interaction of α_2 M-Me with endothelial cells.

Nature of recognition sites for α_2 M on rat liver cells

In order to determine the importance of the scavenger receptor in the interaction of α_2 M with parenchymal, endothelial and Kupffer cells, radiolabelled α_2 M-Me at 2.6 μ g/ml was incubated for 10 min at 37 °C with various concentrations of unlabelled poly(I) or Ac-LDL up to 500 μ g/ml. With 500 μ g of poly(I)/ml the subsequent association of α_2 M-Me with endothelial cells was

Fig. 7. Association of α_2 M-Me with rat parenchymal and endothelial cells

Freshly isolated rat parenchymal (a) and endothelial (b) cells were incubated for 10 min at 37 °C with increasing amounts of ^{125}I -labelled human α_2 M-Me. Cell association was determined, and values are means $\pm$ S.E.M. of two experiments.

nearly completely blocked (5% of control), while association with Kupffer cells was lowered to 25% of the control (Fig. 10a). The association of α_2 M-Me with parenchymal cells was not affected by poly(I), even at a concentration of the competitor of 500 $\mu\text{g/ml}$. Unlabelled Ac-LDL effectively blocked the association of α_2 M-Me with endothelial cells. At 500 μg of Ac-LDL/ml the cell association of α_2 M-Me was lowered to 9% of the control value. With Kupffer cells also the interaction of α_2 M-Me with the cells is mainly scavenger-receptor-mediated, as shown by the effects of Ac-LDL (Fig. 10b). Association of α_2 M-Me with parenchymal cells was not affected by large amounts of Ac-LDL. The competition of unlabelled Ac-LDL with radioiodinated α_2 M-Me for binding to endothelial cells as noted here is similar to results obtained on radiolabelled Ac-LDL binding to endothelial cells [45].

Charge characterization of α_2 M-Me and α_2 M-Tr

To analyse the effect of activation on the charge of α_2 M, the protein was activated by either methylamine or trypsin. After subsequent purification on a Bio-Gel A-1.5m column, the protein was analysed by non-denaturing PAGE. In agreement with earlier publications [4,47], α_2 M was converted from a slow to a so-called fast form which is identical for both trypsin- and methylamine-activated α_2 M (Fig. 11a). In a subsequent experiment we analysed both activated forms of the radioiodinated protein by agarose-gel electrophoresis. It appears that the electrophoretic mobility of α_2 M-Tr radioactivity is identical with that of the unactivated macroglobulin (R_f 0.26). In contrast, activation

Fig. 8. Effect of pH on the association of α_2 M-Me with parenchymal and endothelial cells

Freshly isolated rat parenchymal (a) and endothelial (b) cells were incubated for 10 min at 37 °C with 2.6 μg of ^{125}I -labelled human α_2 M-Me/ml (3.6 nM) at different pH values. Association is expressed as a percentage of the association obtained at pH 7.5, which was for parenchymal and endothelial cells 10.6 ± 2.5 ng and 226 ± 36 ng of α_2 M/mg of cell protein respectively. Values are means of two experiments.

of α_2 M by methylamine resulted in a change in the electrophoretic mobility of the protein (Fig. 11b), whereby a fraction of the α_2 M-Me showed a slower mobility, indicative of a diminished negative charge.

DISCUSSION

Activation of α_2 M results in its increased removal from serum and rapid uptake by the liver. There are, however, disagreements on the liver cell types reported to be responsible for the uptake of activated α_2 M. Ohlsson [19] showed, using electron microscopy, that uptake of bovine α_2 M-Tr is carried out by liver macrophages (Kupffer cells) in adult dogs. With a similar technique, Davidsen *et al.* [6] showed that human α_2 M-Tr is almost exclusively taken up by liver parenchymal cells in rats (88% of the grains counted). A similar result came from Feldman *et al.* [9], studying human α_2 M-Me in mice, although the contribution of the non-parenchymal cells was higher than in the former study (20% of the grains counted). Furthermore, *in vitro* experiments indicated that activated α_2 M can be taken up via receptor-mediated endocytosis by isolated parenchymal cells, adipocytes, fibroblasts or macrophages [3,18,20–23], suggesting that a number of cell types may participate in the uptake of activated α_2 M.

Fig. 9. Effect of Mg-EGTA and Ca^{2+} on the cell association of $\alpha_2\text{M-Me}$ with rat parenchymal and endothelial cells

Freshly isolated rat parenchymal (a) and endothelial (b) cells were incubated for 10 min at 37°C with $2.6\text{ }\mu\text{g}$ of ^{125}I -labelled human $\alpha_2\text{M-Me}$ /ml (3.6 nM) at the indicated concentrations of Mg-EGTA or Ca^{2+} . The cell association is expressed as percentage of the binding obtained at $2\text{ mM-}\text{Ca}^{2+}$. The 100% association values with parenchymal and endothelial cells were $11.3 \pm 2.7\text{ ng}$ and $202 \pm 24\text{ ng}$ $\alpha_2\text{M}$ /mg of cell protein respectively.

Fig. 10. Competition by poly(I) and Ac-LDL with association of $\alpha_2\text{M-Me}$ with rat liver parenchymal, endothelial and Kupffer cells

Freshly isolated rat parenchymal (●), endothelial (▲) and Kupffer (▼) cells were incubated for 10 min at 37°C with $2.6\text{ }\mu\text{g}$ of ^{125}I -labelled human $\alpha_2\text{M-Me}$ /ml (3.6 nM) in the absence or presence of the indicated amount of unlabelled poly(I) (a) or Ac-LDL (b). Cell association is expressed as a percentage of the radioactivity obtained in the absence of competitor. The 100% binding values for ^{125}I -labelled $\alpha_2\text{M-Me}$ to parenchymal, endothelial and Kupffer cells were $13.2 \pm 3.0\text{ ng}$, $234 \pm 45\text{ ng}$ and $98 \pm 22\text{ ng}$ of $\alpha_2\text{M}$ /mg of cell protein respectively. Values are means $\pm$ S.E.M. of two experiments.

Recently we reported that the liver uptake of $\alpha_2\text{M-Me}$ *in vivo* is shared equally between rat liver parenchymal and endothelial cells [12], as shown by isolation of the different cell types after injection of $\alpha_2\text{M-Me}$. The present studies were aimed at characterizing the uptake of $\alpha_2\text{M-Me}$ by the endothelial cells and at comparing the uptake systems for $\alpha_2\text{M-Me}$ in endothelial and parenchymal cells. During these studies we realized that various means of activation of $\alpha_2\text{M}$ may influence its biological properties differently. For this reason we compared the behaviour of methylamine-activated $\alpha_2\text{M}$ with those of trypsin-activated $\alpha_2\text{M}$. The present data clearly show that the method of activation defines the uptake of $\alpha_2\text{M}$ by the different liver cell types in the rat. We confirmed that injection of $\alpha_2\text{M-Me}$ results in an equal uptake of the protein by liver parenchymal and endothelial cells (respectively 53.1% and 48.3% of the liver-associated radioactivity). Since only 3.3% of the total liver protein is present in endothelial cells, the specific uptake/mg of cell protein is 23.8-fold higher for endothelial cells than for parenchymal cells. In contrast, $\alpha_2\text{M-Tr}$ is almost exclusively taken up by parenchymal cells (90.4% of liver-associated radioactivity). The recognition

site for $\alpha_2\text{M-Me}$ on endothelial cells differs from that on parenchymal cells in several respects: (1) parenchymal cells possess a saturable uptake mechanism, while uptake of $\alpha_2\text{M-Me}$ by endothelial cells is not saturable up to $100\text{ }\mu\text{g/ml}$; (2) with parenchymal cells the optimal pH for cell association is 7.5–9.0, whereas with endothelial cells a sharp optimum at pH 7.5 is observed; (3) association of $\alpha_2\text{M-Me}$ with parenchymal cells is strongly diminished in the absence of Ca^{2+} , while association with endothelial cells is not influenced by a variation in the Ca^{2+} concentration; (4) poly(I) and Ac-LDL both greatly decrease the association of $\alpha_2\text{M-Me}$ with endothelial cells, but parenchymal cell-mediated uptake is unaffected. These data lead to the conclusion that the uptake of $\alpha_2\text{M-Me}$ by endothelial cells is mediated by the so-called scavenger receptor [44].

The association of $\alpha_2\text{M-Me}$ with endothelial cells *in vitro* is not saturable up to $100\text{ }\mu\text{g}$ of protein/ml, while with parenchymal cells a clear saturation curve is obtained. *In vivo*, we could not demonstrate any effect of the amount of protein injected on the percentage uptake of $\alpha_2\text{M-Me}$ by endothelial cells and parenchymal cells when we varied the dose between 2 and

Fig. 11. Non-denaturing PAGE and agarose gel electrophoresis of native α_2 M, α_2 M-Me and α_2 M-Tr

After radiolabelling, human α_2 M (activated or unactivated) was purified on a Bio-Gel A-1.5m column (0.7 cm $\times$ 25 cm). (a) Samples of 125 I-labelled human α_2 M (lane 1), 125 I-labelled human α_2 M-Me (lane 2) or 125 I-labelled human α_2 M-Tr (lane 3) (5 μ g of protein) were applied to 5% non-denaturing polyacrylamide gel. After electrophoresis, protein bands were stained with Coomassie Brilliant Blue. (b) 125 I-labelled human α_2 M (Δ), 125 I-labelled human α_2 M-Me ($\triangle$) and 125 I-labelled human α_2 M-Tr ($\bullet$) (2 μ g each) were subjected to agarose gel electrophoresis. After electrophoresis the gel was cut into 0.5 cm fragments and examined for radioactivity.

20 μ g of α_2 M-Me. However, it must be realized that both doses will result in plasma concentrations in the linear part of the *in vitro* saturation curves, so that for both cell types a linear increase in uptake would be expected.

Studies by our group [45] have shown previously that Ac-LDL is taken up *in vivo* mainly by the liver endothelial cells. We have also found that the amount of Ac-LDL receptors on Kupffer cells is not negligible, and on a cell-protein basis is approx. 5-fold lower than on endothelial cells [45]. The relative importance of endothelial and Kupffer cells in the uptake of α_2 M-Me is in accordance with these relative proportions of Ac-LDL receptors.

Our results *in vivo* also show that activation of α_2 M by methylamine leads to the presence of two separate populations. It appears that the form of α_2 M-Me which is recognized by the scavenger receptor is not recognized by the α_2 M receptor on parenchymal cells. Probably, the splicing of the internal thioester bond may lead to the formation of multiple conformations, whereby recognition either by the parenchymal cell receptor (presumably the LDL-related receptor protein) or the scavenger receptor is induced.

Numerous reports have indicated similar effects of the reactions of trypsin and methylamine with α_2 M, as revealed by, for example, non-denaturing PAGE [4,8,13], circular dichroism

[14,15], sedimentation velocity [14,15], ultraviolet absorption [14,15], intrinsic protein fluorescence [16], change in surface structure [17] and kinetics of receptor-mediated endocytosis by macrophages and fibroblasts [4,7,18]. Kaplan *et al.* [18] and Imber & Pizzo [4] showed that rabbit alveolar macrophages and mouse peritoneal macrophages respectively did not distinguish between the receptor-mediated uptake of α_2 M-Me and α_2 M-Tr. Gonias *et al.* [48] reported slight differences between α_2 M-Me and α_2 M-Tr, based on size-exclusion chromatography. These differences could be due to a difference in charge, and as such may support our observations.

The data obtained with agarose-gel electrophoresis demonstrate that methylamine induces a mobility of the protein which differs from that of native α_2 M or trypsin-activated α_2 M. In contrast, non-denaturing PAGE indicates similar mobilities for both α_2 M-Me and α_2 M-Tr. It is generally accepted that the conversion of the slow form of α_2 M into the fast form is caused by a conformational change [47], which leads to a more compact form of the molecule, resulting in a faster mobility on PAGE. This is supported by electron-microscopic studies [49,50]. Agarose-gel electrophoresis is used to analyse the charge of proteins or macromolecules. For example, the oxidation of LDL by copper for various periods of time results in an increased mobility of the lipoprotein on agarose gels, which is an indication of an increased negative charge [45]. However, the size of LDL is not changed during oxidation. We postulate, therefore, that the difference in charge between α_2 M-Tr and α_2 M-Me is responsible for their different behaviours *in vivo* and *in vitro*.

The physiological relevance of the uptake of α_2 M-Me by the scavenger receptor on endothelial cells remains unresolved. The scavenger receptor is known to mediate uptake of Ac-LDL and formaldehyde-treated albumin [44]; neither of these substrates is a normal constituent of the body, however. Recently it has been proposed from studies *in vivo* and *in vitro* that lipid IV_A, a bioactive precursor of lipopolysaccharide from Gram-negative bacteria, might be a substrate for the scavenger receptor [51]. As such, lipid IV_A may be the first naturally occurring ligand for the scavenger receptor described so far. Reaction of α_2 M with proteolytic enzymes such as trypsin remains the most likely form of activation of this molecule. Whether activation of α_2 M by methylamine represents a physiological activation needs to be determined. It is possible that, in the bloodstream or the extracellular space, α_2 M meets small nucleophiles and is activated in a manner similar to that described here. In this case, the scavenger receptor on endothelial liver cells might function as a physiological protection system of the body against activated α_2 M, which is not adequately recognized by the α_2 -M receptor/LRP on parenchymal cells.

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