Intracellular transport of formaldehyde-treated serum albumin in liver endothelial cells after uptake via scavenger receptors

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Endocytosis of formaldehyde-treated serum albumin (FSA) mediated by the scavenger receptor was studied in rat liver endothelial cells. Suspended cells had about 8000 receptors/cell, whereas cultured cells had about 19000 receptors/cell. K_d was 10^{-8} M in both systems. Cell-surface scavenger receptors were found exclusively in coated pits by electron microscopy, by using ligand labelled with colloidal gold. Cell-surface-bound FSA could be released by decreasing the pH to 6.0; it was therefore possible to assess the rate of internalization of surface-bound ligand. This rate was very high: $t_{1/2}$ for internalization of ligand prebound at 4 °C was 24 s. The endocytic rate constant at 37 °C, K_e , measured as described by Wiley & Cunningham [(1982) *J. Biol. Chem.* **257**, 4222–4229], was 2.44 min^{-1} , corresponding to $t_{1/2} = 12 \text{ s}$. Uptake of FSA at 37 °C after destruction of one cell-surface pool of receptors by Pronase was decreased to 60%. This finding is compatible with a relatively large intracellular pool of receptors. The intracellular handling of ^{125}I -tyramine-cellobiose-labelled FSA (^{125}I -TC-FSA) was studied by subcellular fractionation in sucrose gradients, Nycodenz gradients or by differential centrifugation. The density distributions of degraded and undegraded ^{125}I -TC-FSA after fractionation of isolated non-parenchymal cells and whole liver were similar, when studied in Nycodenz and sucrose gradients, suggesting that the subcellular distribution of the ligand was not influenced by the huge excess of non-endothelial material in a whole liver homogenate. Fractionation in sucrose gradients showed that the ligand was sequentially associated with organelles banding at 1.14, 1.17 and 1.21 g/ml. At 9–12 min after intravenous injection the ligand was in a degradative compartment, as indicated by the accumulation of acid-soluble radioactivity at 1.21 g/ml. A rapid transfer of ligand to the lysosomes was also indicated by the finding that a substantial proportion of the ligand could be degraded by incubating mitochondrial fractions prepared 12 min after intravenous injection of the ligand. The results indicate that FSA is very rapidly internalized and transferred through an endosomal compartment to the lysosomes. The endosomes are gradually converted into lysosomes between 9 and 12 min after injection of FSA. The rate-limiting step in the intracellular handling of ^{125}I -TC-FSA is the degradation in the lysosomes.

INTRODUCTION

The sinusoid-lining endothelial cells of the mammalian liver are highly active endocytic cells. These cells have receptors that mediate the endocytic uptake from blood of, for instance, mannose-terminated glycoproteins (Hubbard *et al.*, 1985), glycosaminoglycans (Smedsrød *et al.*, 1984), collagen (Smedsrød *et al.*, 1985) and ceruloplasmin (Kataoka & Tavassoli, 1985). The liver endothelial cells also have a so-called 'scavenger receptor', with broad chemical specificity (Nagelkerke *et al.*, 1983; Blomhoff *et al.*, 1984a,b). Although native serum albumin is not recognized, treatment of albumin with formaldehyde, acetic anhydride or malondialdehyde will convert it into a scavenger ligand (Eskild & Berg, 1984). This receptor also recognizes low-density lipoprotein (LDL) and other proteins after treatment with agents that alter lysine residues: succinic anhydride (Haberland *et al.*, 1984), maleic anhydride (Brown *et al.*, 1980) and malondialdehyde (Fogelman *et al.*, 1980). The scavenger receptor has been found in extrahepatic macro-

phages, in addition to endothelial cells (Brown & Goldstein, 1985). The receptor has been purified from a mouse macrophage cell line, P388D₁, and shown to be a glycoprotein of approx. 260000 Da (Via *et al.*, 1985).

The purpose of this study was to obtain information about binding, uptake and intracellular transport of formaldehyde-treated albumin (FSA) in rat liver endothelial cells. It was found that surface-bound ligand could be released by lowering pH to 6.0; this observation enabled us to estimate the rate of internalization. Intracellular transport was studied by subcellular fractionation in sucrose gradients. FSA labelled with ^{125}I -tyramine-cellobiose (^{125}I -TC-FSA) was injected intravenously and liver samples were removed and fractionated at various time points afterwards. When ^{125}I -TC-FSA is degraded, the labelled acid-soluble degradation products are trapped in the lysosomes in which they are formed (Pittman *et al.*, 1983). Initial experiments confirmed that ^{125}I -TC-FSA is taken up mainly in the endothelial cells of liver. Therefore the labelled degradation products formed from ^{125}I -TC-FSA may serve

Abbreviations used: FSA, formaldehyde-treated serum albumin; TC-FSA; tyramine-cellobiose-labelled FSA; BSA, bovine serum albumin; LDL, low-density lipoprotein; NPC, non-parenchymal liver cells; PBS, phosphate-buffered saline (0.9% NaCl in 20 mM-sodium phosphate, pH 7.4).

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as markers for the lysosomes of the endothelial cells. This is an advantage, since these lysosomes represent only a small fraction of the total lysosomal population in the whole liver homogenate.

MATERIALS AND METHODS

Chemicals

Collagenase (EC 3.4.24.3) and Pronase (type E, from *Streptomyces griseus*) were purchased from Sigma Chemical Co. Human serum albumin was from Kabi, and ^{125}I from New England Nuclear. Nycodenz and Maxidenz were supplied by Nycomed, Oslo, Norway. Tyramine-cellobiose was prepared as described by Pittman *et al.* (1983). Human serum albumin was treated with formaldehyde as previously described (Eskild & Berg, 1984; Mego & McQueen, 1965). FSA was labelled with ^{125}I -tyramine-cellobiose as described by Pittman *et al.* (1983). Specific radioactivity of the ligand was 4×10^3 c.p.m./ng (130 Bq/ng).

Animals and injections

Male Wistar rats (about 200 g) were anaesthetized with phenobarbital intraperitoneally. A dose of 0.8 nmol of ^{125}I -TC-FSA (2.2 MBq) was injected into the right femoral vein.

Preparation of liver cells

(a) Separation of liver cells after intravenous injection of ^{125}I -TC-FSA, in order to determine the distribution of labelled ligand between various cell types. Isolated liver cells were prepared by collagenase perfusion at various times after injection of ^{125}I -TC-FSA (Tolleshaug *et al.*, 1977). The liver cell suspension was first diluted to 200 ml and centrifuged at 50 g for 2 min to sediment the hepatocytes. The hepatocytes were further purified by centrifugal elutriation. The final preparation of hepatocytes contained less than 2% contaminating non-parenchymal cells (NPC), and were >95% viable by the Trypan Blue exclusion test.

The rotor was run at 1500 rev./min, and the cells eluted in the flow range between 20 and 55 ml/min were collected. The cells in the first supernatant were pelleted at 500 g for 2 min and resuspended in 80 ml of incubation buffer (Tolleshaug *et al.*, 1977). Protease was added to 0.5%, and the cells were incubated at 10 °C for 60 min with shaking. Subsequently the NPC were purified by flotation in Nycodenz (Blomhoff *et al.*, 1984d). Contaminating hepatocytes accounted for less than 0.5% of the cells. The yield was 20×10^6 NPC/g of liver on average. The NPC were separated into an endothelial-cell and Kupffer-cell fraction by centrifugal elutriation (Blomhoff *et al.*, 1984c).

(b) Isolation of sinusoidal endothelial cells for studies of endocytosis *in vitro*. NPC were prepared from a total liver-cell suspension after selective permeabilization of the parenchymal cells by enterotoxin from *Clostridium perfringens*, as described by Blomhoff *et al.* (1984d).

For experiments with suspended cells, the liver endothelial cells were isolated further by a two-step centrifugal elutriation procedure (Blomhoff *et al.*, 1984c).

To obtain cultures of endothelial cells, the NPC were suspended in medium RPMI 1640 supplemented with 100 µg of penicillin/ml and 50 µg of streptomycin/ml. The cells were seeded in Costar wells (16 mm) or dishes

(diameter 35 mm or 60 mm) coated with fibronectin. Seeding density was 0.5×10^6 NPC/cm² in 0.25 ml of RPMI/cm². Approx. 90 min after seeding the non-adherent cells were removed, followed by washing of the cultures. Fresh medium was added and incubation of the cells was continued for another 30–60 min at 37 °C in a humidified atmosphere containing 5% CO₂ in air.

The cell preparations (suspensions and cultures) consisted of 85–95% endothelial cells, 3–10% Kupffer cells and 2–5% unidentified cells (Smedsrød *et al.*, 1982).

Binding assays

Suspended cells. Endothelial cells (10^7 /ml) were incubated with ligand at 4 °C for 75 min until equilibrium. After incubation, triplicate 500 µl samples were transferred to 4.5 ml of ice-cold incubation medium. Cells were pelleted at 400 g for 3½ min and washed twice in incubation medium. Cell-associated radioactivity at 75 min was corrected for non-specific binding, measured in the presence of labelled ligand and 2 µM unlabelled ligand.

Cells in monolayer. The cells were incubated on a rocking platform until equilibrium, for 6 h at 4 °C, in 35 mm dishes in 600 µl of RPMI with 1% BSA and various concentrations of ^{125}I -FSA. The cells were subsequently washed with 3×1 ml of PBS and 3×1 ml of PBS with 1% BSA before being dissolved in 1 ml of 1% SDS in 0.3 M-NaOH. Non-specific binding was determined in the presence of 2 µM unlabelled FSA.

Release of surface-bound ligand by lowering pH

Suspended cells. Endothelial cells in suspension were incubated with ^{125}I -FSA at 4 °C until equilibrium. Then samples of cells were transferred to 10 vol. of ice-cold buffer, pH ranging from 7.5 to 5.5. Hepes (20 mM) was used as buffering agent at pH 7.5 and pH 7.0, Mops (20 mM) at pH 6.75 and Mes (20 mM) at pH 6.5, 6.0 and 5.5. After 10 min on ice, the cells were washed free of unbound ligand by centrifugation at 400 g for 3½ min. Incubation buffer with pH 7.5 was used to suspend the cells in the second and third wash.

Cultured cells. Ligand was bound to cells in 60 mm dishes as described above. After incubation the cells were washed with 3×1.5 ml of PBS, followed by 3×1.5 ml of PBS+1% BSA. Then duplicate dishes were incubated on a rocking platform at 4 °C with 2 ml of incubation buffer, containing 1% BSA, at various pH values as described for suspended cells. After 10 min the cells were washed with 3×1.5 ml of PBS before being dissolved in 2 ml 1% SDS in 0.3 M NaOH.

Incubation of cultured liver endothelial cells with gold-labelled FSA

The cultured liver endothelial cells were incubated in 35 mm dishes with 0.40 µM-Au-FSA in 600 µl of RPMI containing 1% BSA. Non-specific binding was measured in control dishes to which 10 µM-unlabelled FSA had been added together with Au-FSA. The cells were incubated at 4 °C for 6 h on a rocking platform. After incubation, the cells were washed as described above.

Transmission electron microscopy

After fixation in 2.5% (v/v) glutaraldehyde, the cells were processed for electron microscopy essentially as

described by Brunk *et al.* (1971). Briefly, the specimen was dehydrated in a graded series of ethanol solutions and then in propylene oxide, which has the additional effect of dissolving the plastic culture dishes. The resulting film of cells in a monolayer was washed and infiltrated with Epon. Ultrathin sections were studied in a Philips EM 410 transmission electron microscope.

Uptake and degradation of FSA by endothelial cells

This was studied at 37 °C by determination of cell-associated radioactivity and acid-soluble radioactivity in the medium. Cell-associated ^{125}I -FSA was measured after separating the cells from the incubation medium either by centrifugation through an oil mixture (Nilsson & Berg, 1977) when using suspended cells or by washing the cultures with 6×1 ml of PBS. Degradation of ^{125}I -labelled FSA leads to formation of acid-soluble radioactive products that leave the cells rapidly. Hence total uptake of ligand was calculated as the sum of cell-associated and acid-soluble ligand at a given time point.

Homogenization procedures

Isolated cells. NPC from one liver, suspended in 8–10 ml of 0.25 M-sucrose/1 mM-Hepes (pH 7.2)/1 mM-EDTA, were homogenized in a LOX-press by a modification of the method described by Tagesson *et al.* (1973). It was found that 80–90 % of the cells were disintegrated at an operating pressure of 1.5 MPa (15 atm). Before removal of the N-fraction by centrifugation at 2000 *g* for 2 min, the homogenate was subjected to five strokes with the tight-fitting pestle in a Dounce homogenizer. The N-fraction was resuspended in 5–6 ml of homogenization buffer and homogenized a second time. This homogenate was treated as above, and the two supernatants were pooled and termed post-nuclear fraction (CE). The quality of the homogenate was evaluated by measuring the proportion of β -glucuronidase (Gianetto & de Duve, 1955) that was latent. Free activity of the enzyme was measured by incubation at 37 °C for 10 min in 0.25 M-sucrose, 10 mM-acetate buffer, pH 5.0, and 1 μM -phenolphthalein glucuronide. Total activity was determined in the presence of 0.1 % Triton X-100. The latent β -glucuronidase activity was found to be above 70 % of total activity in the endothelial-cell homogenates.

Whole liver. Liver lobules were tied off at indicated time points and transferred to ice-cold 0.25 M-sucrose/1 mM-Hepes (pH 7.2)/1 mM-EDTA and minced with scissors. Homogenates (10 %, w/v) were prepared in the same buffer by using five strokes with a loose-fitting pestle and five strokes with a tight-fitting one in a Dounce homogenizer. Post-nuclear fractions were prepared by centrifugation as above.

Fractionation of post-nuclear fractions on Nycodenz and sucrose gradients

Preformed linear Nycodenz and sucrose gradients were prepared in 38 ml Ultra-clear tubes (Beckman) by using a two-chamber mixer as described by Kindberg *et al.* (1984).

A portion (4 ml) of CE was loaded on top of each gradient. Centrifugation was carried out at 85000 *g* for 2 h (Nycodenz gradients) or 3 h (sucrose gradients) at 4 °C. Subsequently, the gradients were fractionated into

2 ml fractions by upward displacement with Maxidens. Density of fractions was determined by measurement of the refractive index (Rickwood, 1983). The distribution of marker enzymes for lysosomes (β -acetylglucosaminidase; Barrett, 1972) and plasma membrane (5'-nucleotidase; El-Aaser & Reid, 1969) were determined in gradients of purified NPC only.

Fractionation of liver homogenate by differential centrifugation

A nuclear fraction was sedimented by centrifugation at 2000 *g* for 2 min. The large-granule fraction (ML) and microsomal fraction (P) were sedimented by centrifugation at 25000 *g* for 9 min and at 48000 *g* for 70 min respectively.

Degradation of particle-bound ^{125}I -TC-FSA

At various time points after intravenous injection of ^{125}I -TC-FSA, ML fractions of whole-liver homogenates were prepared by differential centrifugation and incubated at 37 °C in 0.25 M-sucrose/10 mM-acetate buffer, pH 5.0, in the presence or the absence of 0.1 % Triton X-100. At the indicated time points, duplicate 1 ml samples were removed for determination of acid-soluble radioactivity by precipitation in 10 % (v/v) trichloroacetic acid.

RESULTS

Receptor number on suspended and cultured sinusoidal endothelial cells

Suspended and cultured endothelial cells were incubated at 4 °C until equilibrium with increasing concentrations of ^{125}I -FSA. Fig. 1 shows Scatchard (1949) plots of the binding data. For suspended cells the receptor number was found to be approx. 8000 receptors/cell.

Fig. 1. Concentration-dependence of the binding of FSA to endothelial cells

Endothelial cells in suspension (●) or in culture (○) (35 mm dishes) were incubated for 75 min and 6 h, respectively, at 4 °C with 10–500 nM- ^{125}I -FSA. The data for cell-bound ligand were corrected for non-specific binding as outlined in the text and are presented as Scatchard (1949) plots.

Fig. 2. Distribution of surface-bound gold-labelled FSA on cultured liver endothelial cells

Liver endothelial cells in monolayer culture were incubated with 400 nm-Au-FSA at 4 °C for 6 h. After incubation, unbound Au-FSA was removed by washing, and the cells were processed for transmission electron microscopy as described in the Materials and methods section. Magnification $\times 9600$.

The dissociation constant, K_d , was calculated to be 10^{-8} M. The cells in monolayer were found to have 19000 receptors/cell, whereas the K_d was unchanged.

Scavenger receptors are located in coated pits

Gold-particle-labelled FSA was allowed to bind at 4 °C to monolayer cultures of endothelial cells. After removal of unbound ligand, the cells were processed for transmission electron microscopy.

Fig. 2 shows cross-sectioned endothelial cells. Gold particles are found in coated-pit regions exclusively. In the presence of excess unlabelled FSA no Au-FSA bound to the cells (results not shown).

Effect of pH on binding of FSA to liver endothelial cells

Suspended cells with prebound ^{125}I -FSA were exposed to lower pH as described in the Materials and methods section. Fig. 3(a) shows that at pH 6.0 more than 90 % of cell-associated ligand has been released. The same effect of lowering pH was found for FSA binding to endothelial cells in monolayer culture (results not shown). Cell viability was not affected by the low-pH treatment, as judged by the ability to exclude Trypan Blue.

Rate of internalization of ligand

Suspended cells were incubated at 4 °C with ^{125}I -FSA until equilibrium, followed by removal of unbound ligand by washing. Then the temperature of the incubation buffer was rapidly increased to 37 °C by addition of several volumes of warm buffer. After various periods of time, the temperature was quickly lowered to less than 10 °C by addition of 10 vol. of ice-cold buffer, pH 6.0. After 10 min, released ligand was removed. The remain-

ing cell-associated radioactivity was considered intracellular. Fig. 3(b) shows that FSA is internalized very rapidly. The apparent $t_{1/2}$ for the internalization was found to be 24 s on average.

The $t_{1/2}$ is related to the rate constant for internalization by the equation $K_e = \ln 2/t_{1/2}$ (Wiley & Cunningham, 1982). Our data therefore indicate that $K_e = 1.73 \text{ min}^{-1}$. This value, however, is a minimum value, since cells may need some time to re-establish the cytoskeleton when transferred from 4 °C to 37 °C. We therefore wished to determine the rate of internalization at 37 °C. This was done in accordance with Wiley & Cunningham (1982). Cells in monolayer or suspension were incubated at 37 °C in the continued presence of 0.8 nM- ^{125}I -FSA. At selected time points, internalized and surface-bound ligand were determined as described in the Materials and methods section. Fig. 3(c) shows the increase in the ratios between internalized and surface-bound ligand as a function of time in cultured cells. The rate constant for internalization, K_e , which is equal to the slope of this curve, was found to be $2.44 \pm 0.20 \text{ min}^{-1}$ (S.D., $n = 4$). From this result $t_{1/2}$ is calculated to be 12 s. Very similar results (not shown) were obtained for suspended cells.

Endothelial cells have an intracellular pool of scavenger receptors

The very rapid internalization of FSA by the liver endothelial cells suggests the existence of an intracellular pool of receptors. Evidence for such a pool was sought by studying the recovery of the binding capacity after destruction of the surface pool of receptors by treatment with Pronase at 10 °C for 10 min. Pronase destroys the receptor, but at this temperature no receptor replacement

Fig. 3. Binding and uptake of FSA by endothelial cells

(a) Effect of pH of the medium on binding of FSA to endothelial cells. Endothelial cells in suspension were incubated for 75 min at 4 °C in the presence of 10 nM- 125 I-FSA. Non-specific binding was measured in the presence of 2 μ M-FSA. After incubation, samples of the cell suspension were transferred to 10 vol. of ice-cold buffer of various pH values as indicated in the Figure. The cells were washed free of unbound ligand after 10 min. Cell-bound radioactivity was corrected for non-specific binding and presented as a percentage of control values. FSA bound to endothelial cells at pH 7.4 was used as control. (b) Internalization of surface-bound FSA. Endothelial cells in suspension were incubated at 4 °C with 25 nM- 125 I-FSA. After 75 min, unbound ligand was removed. The temperature was increased to 37 °C and, after the time periods indicated in the Figure, lowered to 4 °C again. Simultaneously, pH was changed to 6.0. The remaining surface-bound 125 I-FSA was allowed to dissociate for 10 min. Dissociable radioactivity is presented as a percentage of total radioactivity (c.p.m.) in each sample. (c) Rate constant for uptake of FSA by endothelial cells. Cultured endothelial cells (60 mm dishes) were incubated in the presence of 0.8 nM- 125 I-FSA for 3–15 min at 37 °C. After incubation the cells were cooled to 4 °C and unbound ligand was removed by six washes. The cells were then incubated at 4 °C with buffer, pH 6.0, for 10 min to dissociate surface-bound ligand. The ratio of internalized to released radioactivity was plotted against time. The slope of the curve was determined by linear regression, giving $K_e = 2.44 \pm 0.20 \text{ min}^{-1}$, with a correlation coefficient of 0.98.

Fig. 4. Effect of Pronase on FSA binding and uptake

(a) Recovery of binding capacity after Pronase treatment at 10 °C. Endothelial cells in suspension were preincubated without (●) or with 0.25 % (○) Pronase at 10 °C for 60 min, and then washed free of Pronase and incubated at 37 °C for the times indicated. The cells were then incubated with 10 nM- 125 I-FSA for 75 min at 4 °C. Non-specific binding was determined in the presence of 2 μ M-FSA. (b) Uptake of FSA by endothelial cells after treatment with Pronase at 10 °C or 37 °C. Endothelial cells in suspension were preincubated with 0.25 % Pronase for 60 min at 10 °C (■) or for 30 min at 37 °C (▼). Control cells (●) were preincubated in the absence of Pronase. After removal of Pronase by washing, the cells were incubated at 37 °C with 1 nM- 125 I-FSA. Uptake of 125 I-FSA is presented as a percentage of total radioactivity in the suspension.

can take place. The cells were washed free of Pronase and incubated for various time periods at 37 °C before determination of the binding capacity at 4 °C.

Fig. 4(a) shows that the endothelial cells rapidly recover their maximal binding capacity. After re-incubation for 10 min at 37 °C, the Pronase-treated cells bound the same amount of ligand as did control cells.

The effect of eliminating one surface pool of receptors on ligand uptake at 37 °C was tested. Fig. 4(b) shows that cells treated with Pronase at 10 °C took up about 60–70 % of the amount of ligand taken up by control cells during a 40 min incubation. For comparison, cells that had been treated with Pronase at 37 °C for 30 min were included in these experiments. Such cells did not take up any FSA.

Table 1. Distribution of ^{125}I -TC-FSA among liver cell types at various time points after intravenous injection

A liver cell suspension was prepared by collagenase perfusion at the indicated time points after intravenous injection of ^{125}I -TC-FSA. Hepatocytes were purified by differential centrifugation and centrifugal elutriation. NPC were purified by Pronase treatment, followed by centrifugal elutriation. Peroxidase staining was used to discriminate Kupffer cells and endothelial cells. The data are average values ($\pm$ S.D.) of duplicate determinations from three separate experiments; n.d., not determined.

Cells	Total radioactivity for each cell type (% of total liver cell radioactivity)				
	10 min	30 min	60 min	4 h	24 h
Hepatocytes	32.0 $\pm$ 5.5	32.8 $\pm$ 3.3	23.7 $\pm$ 1.0	23.1 $\pm$ 2.5	22.0 $\pm$ 6.2
NPC	68.0 $\pm$ 5.5	67.2 $\pm$ 4.5	76.3 $\pm$ 3.0	76.9 $\pm$ 3.3	78.0 $\pm$ 7.0
Endothelial cells	n.d.	62.6 $\pm$ 4.5	71.7 $\pm$ 13.6	69.3 $\pm$ 2.0	75.1 $\pm$ 2.2
Kupffer cells	n.d.	4.6 $\pm$ 0.9	5.2 $\pm$ 1.1	7.6 $\pm$ 2.2	2.9 $\pm$ 0.8

Fig. 5. Fractionation of NPC and liver homogenates in Nycodenz (a-c) and sucrose (d-f) gradients

Postnuclear fractions were prepared from liver and isolated NPC, 30 min after intravenous injection of 0.8 nmol of ^{125}I -TC-FSA. The postnuclear fractions were layered on top of linear Nycodenz and sucrose gradients and centrifuged at 85000 g for 2 h or 3 h at 4 $^{\circ}\text{C}$, respectively. After fractionation into 2 ml fractions, acid-precipitable ($\bullet$) and acid-soluble ($\circ$) radioactivity in each fraction were determined. Fractionation of whole liver homogenate is shown in panels (a) and (d), that of NPC homogenates in panels (b) and (e), and the distributions of marker enzymes ($\bullet$, 5'-nucleotidase; $\blacktriangle$, β -acetylglucosaminidase) in NPC gradients are shown in panels (c) and (f). Enzyme activities and radioactivities are plotted against the density of the fractions, except for the three top fractions containing material which does not enter the gradient.

Cellular distribution of ^{125}I -TC-FSA in the liver after intravenous injection

The intracellular transport of ^{125}I -TC-FSA was studied by means of subcellular fractionation of whole liver after intravenous injection of the labelled ligand. It was therefore important to verify that the bulk of the injected ^{125}I -TC-FSA really was sequestered in the liver endothelial cells. Earlier experiments have shown that ^{125}I -labelled FSA is selectively taken up in the hepatic endothelial cells (Blomhoff *et al.*, 1984a). If degradation of ^{125}I -TC-FSA takes place mainly in the endothelial cells, then the labelled degradation products, which are trapped in the lysosomes, may serve as ideal markers for these organelles in the endothelial cells. Table 1 shows the results of an experiment in which the distribution of intravenously injected ^{125}I -TC-FSA was determined in various types of liver cells. Liver cells were prepared by collagenase perfusion at various times after injection of the ligand, and the parenchymal cells, the Kupffer cells and the endothelial cells were separated as described in the Materials and methods section. To calculate the total amount of ligand taken up in each cell type it was assumed that the liver contains 125×10^6 , 19×10^6 and 36×10^6 parenchymal, Kupffer and endothelial cells per g respectively (Blomhoff *et al.*, 1984c; Munthe-Kaas *et al.*, 1976). The results obtained indicate that most of the ^{125}I -TC-FSA was taken up in the endothelial cells. However, some ligand (22–33 %) was also associated with the parenchymal cells. Some of this ligand may be due to aggregated NPC sedimenting in the parenchymal-cell fraction. The uptake by fluid-phase endocytosis is probably negligible. The cellular distribution was independent of time, and >90 % of the radioactivity stayed with the cells for at least 24 h.

Density distribution of ^{125}I -TC-FSA after fractionating NPC or whole-liver homogenates in Nycodenz or sucrose gradients

As some ^{125}I -TC-FSA seemed to be taken up in the parenchymal cells, we compared the subcellular distribution of labelled ligand in isolated NPC and whole-liver homogenates, to determine whether the distribution in endothelial cells was influenced significantly by the huge excess of parenchymal material in the whole-liver homogenate.

To compare the density distribution of degraded and undegraded ^{125}I -TC-FSA for whole liver with that of isolated NPC, liver homogenates and NPC homogenates were prepared from the same liver. At 30 min after intravenous injection of 0.8 nmol of labelled ligand, liver perfusion was started. One liver lobe was removed during the pre-perfusion in Ca^{2+} -free buffer, before collagenase treatment of the rest of the liver. In this way, the isolated cells had continued processing the ligand for about 20 min after removal of the liver lobe.

Fig. 5 shows that the density-distribution curves for degraded and undegraded ligand were similar for whole liver and isolated NPC when fractionated in either Nycodenz or sucrose gradients. In Nycodenz gradients the radioactivity peak was found at 1.12 g/ml, whereas in sucrose gradient the corresponding peak was at 1.21 g/ml. The positions of the radioactivity peaks coincided with the peak for the lysosomal marker enzyme β -acetylglucosaminidase in isolated NPC (Figs. 5c and 5f). Under the conditions of this experiment, all

Fig. 6. Fractionation of liver homogenates in sucrose gradients to study subcellular distribution of ^{125}I -TC-FSA

Postnuclear fractions of liver homogenates were prepared 1 (●), 3 (○), 6 (■), 9 (□), 12 (▼) and 24 (▽) min after intravenous injection of 0.8 nmol of ^{125}I -TC-FSA and layered on top of linear sucrose gradients. After centrifugation at 85000 g for 3 h at 4 °C, acid-precipitable (a) and acid-soluble (b) radioactivities were determined in the fractions. Total acid-precipitable (●) and acid-soluble (○) radioactivity in each fraction are presented as functions of fraction density. Enzyme activities and radioactivities were plotted against the density of the fractions, except for the two top fractions containing material which does not enter the gradient. Arrows indicate the three activity peaks mentioned in the text.

ligand has probably reached the lysosomes, and the density distribution of undegraded and degraded ligand will therefore coincide in the gradients. As expected, more ligand was degraded in the isolated cells, but the distribution curves for cells and whole liver showed peaks at the same positions.

Fractionation of liver homogenates prepared at various time points after intravenous injection of ^{125}I -TC-FSA

Fractionation of liver homogenates prepared after intravenous injection of ^{125}I -TC-FSA showed that, in

both Nycodenz and sucrose gradients, the ligand was associated with structures that increased in density with time after injection. These changes were more pronounced in sucrose gradients, and only results obtained with this gradient system are presented here. Fig. 6 shows the distribution in sucrose gradients of acid-soluble and acid-precipitable radioactivity after fractionation of liver homogenates prepared 1, 3, 6, 9, 12 and 24 min after intravenous injection of ^{125}I -TC-FSA. Three main bands of acid-precipitable radioactivity could be discerned. Very early after uptake had been initiated (<1 min), a main radioactivity peak was seen at 1.14 g/ml in the sucrose gradient. Traces of this band were also seen (as shoulders) in the distribution curves after 3 min and 6 min and may be due to continued uptake of ligand by the liver. Already at 1 min after injection, however, a second peak at 1.17 g/ml could be seen in the distribution curve. This peak increased in size for homogenates fractionated 3, 6 and 9 min after injection of ^{125}I -TC-FSA. Between 9 min and 12 min the ligand reached a third band in the gradient at 1.21 g/ml. Concurrent with the appearance of ^{125}I -TC-FSA in the third band, degradation of the ligand started. The acid-soluble labelled degradation products accumulated in an organelle which banded in the gradient at 1.21 g/ml.

The distribution of acid-soluble radioactivity suggested that the lysosomes of the endothelial cells are slightly denser than the average hepatic lysosome, which shows a peak at 1.20 g/ml in sucrose gradients (results not shown).

Distribution of ^{125}I -TC-FSA in subcellular fractions obtained by differential centrifugation. Liver lobes were removed at various time points after intravenous injection of ^{125}I -TC-FSA, as indicated in Fig. 7. The liver homogenates were fractionated by differential centrifugation into a nuclear fraction (N), a large-granule fraction (ML), a microsomal fraction (P) and a soluble fraction (S). The main changes in the distribution of ligand were seen in the ML fraction and the P fraction; ligand was transferred to the ML fraction from the P fraction. At

Fig. 7. Subcellular distribution of ^{125}I -TC-FSA in liver homogenates after differential centrifugation

Liver homogenates were prepared 1, 3, 6, 9, 12, 24 and 48 min after intravenous injection of 0.8 nmol of ^{125}I -TC-FSA. The homogenates were fractionated by differential centrifugation to give a nuclear fraction (Δ), a large-granule fraction (●), a microsomal fraction (○) and a soluble fraction (▼). Total radioactivity is presented as percentage of total recovered radioactivity in the fractions (at each time point).

12 min after injection, about 70 % and 20 % of the total radioactivity in the homogenate were in the ML fraction and the P fraction respectively.

Degradation of ^{125}I -TC-FSA in the large-granule fraction *in vitro*

To find out whether the transfer of ^{125}I -TC-FSA from the P fraction to the ML fraction was paralleled by an exposure of ligand to lysosomal enzymes, we incubated ML fraction at 37 °C and pH 5 and measured formation of acid-soluble radioactivity *in vitro*. Fig. 8 shows that no degradation *in vitro* took place in the particles prepared 3 min or 6 min after intravenous injection of ^{125}I -TC-FSA. In ML fraction prepared 9 min after injection, some increase in acid-soluble radioactivity was observed during incubation *in vitro*. In the ML fraction prepared after 12 min, the percentage of acid-soluble radioactivity at the beginning of the incubation *in vitro* was higher than at previous time points. After 90 min incubation at pH 5.0, about 30 % of total radioactivity was acid-soluble. It is likely that this value is an underestimation of the actual proteolysis in the lysosomes, since a large proportion of the lysosomes will rupture during incubation at pH 5.0.

DISCUSSION

Sinusoidal endothelial cells brought into suspension round up by somehow retracting their fenestrae, which

Fig. 8. Degradation of ^{125}I -TC-FSA in the large-granule fraction *in vitro*

Large-granule fractions were prepared from liver homogenates obtained 3 min (●, ○), 6 min (▲, △), 9 min (■, □) and 12 min (◆, ◇) after intravenous injection of 0.8 nmol of ^{125}I -TC-FSA. Incubation was carried out at 37 °C in 0.25 M-sucrose/10 mM-sodium acetate, pH 5.0, in the presence (○, ◇) and absence (●, ▲, ■, ◆) of 0.1 % Triton X-100. After various time points, acid-soluble and acid-precipitable radioactivity were determined. Acid-soluble radioactivity is presented as percentage of total radioactivity in each large-granule fraction as a function of time.

appear as a network of vacuoles in the cytoplasm (Drochmans *et al.*, 1977). The higher number of scavenger receptors on cultured endothelial cells (19000/cell) compared with suspended cells (8000/cell) may be due to the larger surface area in cultured cells. The suspended cells with their retracted fenestrae may have transferred scavenger receptors as part of the cell membrane to an intracellular location.

The Pronase experiments showed that the loss of one surface pool of receptors only led to a 30–40% decrease in the amount of ligand taken up during a 40 min incubation at 37 °C without a preceding recovery period. This result suggests the presence of a large intracellular pool of receptors.

Similar results have been reported for the mannose receptor on macrophages, where the loss of one surface pool of receptors led to a 30% decrease in the uptake capacity (Stahl *et al.*, 1980).

The distribution of scavenger receptors on liver endothelial cells was studied by electron microscopy by using gold-labelled FSA. All gold particles were bound in coated pits. This is in agreement with others (Mommaas-Kienhuis *et al.*, 1985; Pitas *et al.*, 1985). Pitas *et al.* (1985), using ¹²⁵I-labelled acetyl-LDL as a ligand for liver scavenger receptors, suggest that the exclusive location of these receptors in coated pits may indicate very rapid transfer from the place of insertion in the plasma membrane to the coated pit. Further, the receptors must be transferred to coated pits in the absence of ligand, supporting the notion of receptor recycling occurring without prior binding of ligand. In contrast with the present data, Traber *et al.* (1983) reported that the scavenger receptors on monocyte-derived macrophages were distributed diffusely in the cell membrane.

We found, in agreement with Horiuchi *et al.* (1985), that binding of ligand to the scavenger receptor was very sensitive to pH of the medium. This result demonstrates the importance of charge in the binding of ligand to the scavenger receptor.

The possibility to distinguish between surface-bound and internalized ligand was used to evaluate the rate of internalization. The value found for the endocytic rate constant at 37 °C ($K_e = 2.44 \text{ min}^{-1}$) was very high compared with other data reported. Table 2 presents data for

K_e in other endocytic systems. Except for the mannose receptor, these receptors operate at a $K_e < 1.0 \text{ min}^{-1}$. In the endothelial cells the high K_e of the scavenger receptor compensates for the relatively low receptor number. This results in the uptake of approximately as many ligand molecules per min as for the galactose receptor on hepatocytes, which have about 3×10^5 receptors per cell (Tolleshaug, 1981).

Fractionation of liver in sucrose gradients after uptake of ¹²⁵I-TC-FSA suggested that the labelled ligand was sequentially associated with three cell compartments with increasing density. These three compartments most likely correspond to (a) coated vesicles, (b) endosomes and (c) lysosomes. Since FSA is selectively bound in coated pits, some of the 'light' structures with which the ligand was initially associated may be coated-pit regions of the plasma membrane. However, the rate of internalization of ligand is so high that a major fraction of the cell-associated ligand after 1 min must reside in coated vesicles. In accordance with our data, De Bruyn *et al.* (1983) found that ferritin endocytosed *in vivo* in rat liver appeared in coated vesicles already 30 s after intravenous injection.

The ligand banding in the gradient at 1.17 g/ml is probably in a pre-lysosomal stage, an endosome, since no degradation of ligand *in vitro* could be demonstrated in particulate fractions prepared up to 9 min after injection. The present data suggest that FSA was transferred very rapidly from endosomes to lysosomes in liver endothelial cells. From 6 to 12 min after injection the ligand was transferred almost quantitatively from vesicles banding at 1.17 g/ml (endosomes) to vesicles banding at 1.21 g/ml (lysosomes). More than 30% of the ligand present in the large-granule fraction 12 min after injection was degraded *in vitro*, whereas no degradation took place in particles prepared 6 min after injection. This suggests that a large proportion of the ligand became accessible for lysosomal enzymes between 6 and 12 min after injection.

The rapid transfer from endosomes to lysosomes occurs not only for FSA, but also for ligands taken up in endothelial cells by the mannose receptor. Quintart *et al.* (1983) gave galactosylated and mannosylated albumin to rats by intravenous injection. At 10 min after injection, subcellular fractionation showed that the mannosylated

Table 2. Rate of internalization for receptor–ligand complexes in different endocytic systems

Data are compiled from the literature. Abbreviations: EGF, epidermal growth factor; apo, apolipoprotein.

Ligand	Receptor	Cell type	K_e	$t_{1/2}$	Reference
FSA	Scavenger	Sinusoidal endothelial	2.44 ± 0.20	17 s*	The present work
Man ₆ -albumin	Mannose	Alveolar macrophages	1.80 ± 0.67	23 s*	Hoppe & Lee (1983)
EGF	EGF	Fibroblasts	0.35–0.69	1–2 min	Carpenter & Cohen (1976)
Transferrin	Transferrin	Hep G ₂	0.20*	3.5 min	Ciechanover <i>et al.</i> (1983)
Transferrin	Transferrin	HeLa	0.14*	5 min	Bleil & Bretscher (1982)
LDL	Apo B,E	Fibroblasts	0.23*	3 min	Goldstein & Brown (1977)
Asialo-orosomucoid	Galactose	Hep G ₂	0.46	2.2 min	Schwartz <i>et al.</i> (1982)
Asialo-orosomucoid	Galactose	Hepatocytes	0.098–0.22	3–7.1 min	Weigel & Oka (1982); Steer & Ashwell (1980); Bridges <i>et al.</i> (1982); Tolleshaug (1981); Conolly <i>et al.</i> (1983)

* Calculated by the equation $K_e = \ln 2/t_{1/2}$.

albumin was associated with the denser lysosomes, whereas galactosylated albumin equilibrated in the low-density region of the gradient, where the hepatocyte endosomes equilibrate. A consequence of this rapid transfer of ligand to the lysosomes is that undegraded ligand accumulates in the lysosomes, indicating that the rate-limiting step in the intracellular degradation of FSA is within the lysosomes. Hence, in the continued presence of high extracellular concentrations of ligand, one would expect that the lysosomes became gradually 'constipated'. This has been reported to be the case after intravenous injection of a large amount of invertase (Jadot *et al.*, 1985). Invertase which is taken up in liver endothelial cells accumulates in the lysosomes to such an extent that they become denser in a sucrose gradient.

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