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Endocytosis of formaldehyde-treated serum albumin via scavenger pathway in liver endothelial cells

Rune BLOMHOFF, Winnie ESKILD and Trond BERG
Institute for Nutrition Research, School of Medicine, University of Oslo, P.O. Box 1046, Blindern, Oslo 3, Norway

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Denatured or modified proteins (including albumin and low-density lipoprotein) are catabolized *in vitro* via scavenger receptors. We have studied the distribution of formaldehyde-denatured albumin in rat liver cells after intravenous injection of tracer doses of the protein. At 12min after injection, most of the formaldehyde-denatured albumin (about 70% of the injected dose) was recovered in liver endothelial cells. Furthermore, isolated liver endothelial cells in suspension and in surface culture took up formaldehyde-denatured albumin by receptor-mediated endocytosis. Our data indicate that the scavenger receptor in liver is mainly located on the endothelial cells. Implications for the catabolism of low-density lipoproteins are discussed.

Denatured and many chemically modified proteins injected intravenously into mammals are rapidly cleared from the circulation (Mego *et al.*, 1967; Brown *et al.*, 1980). Studies *in vitro* have shown that denatured proteins are taken up by cells belonging to the reticuloendothelial system (Buys *et al.*, 1975; van Berkel *et al.*, 1982). These proteins include formaldehyde- (Moore *et al.*, 1977; Wandel *et al.*, 1982) or nitroguanidine- (Buys *et al.*, 1975) treated albumin, maleylated albumin (Goldstein *et al.*, 1979), malonaldehyde-treated LDL (Fogelman *et al.*, 1980) and acetylated (Goldstein *et al.*, 1979) or maleylated (Goldstein *et al.*, 1979) LDL. The main organ responsible for the rapid plasma clearance is the liver (Mego *et al.*, 1967; Nilsson & Berg, 1977; van Berkel *et al.*, 1982).

Several studies suggest that the hepatic receptors that mediate the endocytosis of these proteins are located on the non-parenchymal liver cells (Nilsson & Berg, 1977), and that the binding sites recognize proteins with increased negative charge (Goldstein *et al.*, 1979; Brown *et al.*, 1980). These receptors are called scavenger receptors (Brown *et al.*, 1980; Eskild & Berg, 1982). In addition to the affinity for denatured or chemically modified proteins, the receptors also bind different polyanions, such as certain polysaccharides (dextran sulphate and fucoidin), certain polynucleotides (such as polyinosinic acid and polyguanylic acid) and polyvinyl sulphate (Brown *et al.*, 1980).

Macrophages isolated from a variety of organs exhibit scavenger receptors (Goldstein *et al.*, 1979; Fogelman *et al.*, 1980). It was therefore suggested that the liver macrophages (the Kupffer cells) were responsible for the rapid clearance of denatured proteins from plasma. However, workers in our laboratory (Bøyum *et al.*, 1983) and others (Nagelkerke *et al.*, 1982) have obtained preliminary data suggesting that formaldehyde-treated albumin and acetylated LDL are taken up in liver by the endothelial cells. In the present paper we present data that demonstrate that a scavenger receptor is present on the liver endothelial cells; intravenously injected FHSA is mainly taken up by the endothelial cells in liver. Furthermore, isolated liver endothelial cells in suspension and in surface cultures effectively take up and degrade formaldehyde-treated albumin.

Abbreviations used: FHSA, formaldehyde-treated human serum albumin; HSA, human serum albumin; LDL, low-density lipoprotein.

Materials and methods

Chemicals

[15(n)-^{3}H]Retinol (all-*trans*) and Na^{125}I were obtained from New England Nuclear, Boston, MA, U.S.A., and Amersham International, Amersham, Bucks., U.K., respectively. Percoll was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Nycodenz and fluorescein amine-conjugated ovalbumin were generously donated by Nyegaard and Co. Oslo, Norway, and Dr. B.

Smedsrød, Institute of Medical and Physiological Chemistry, The Biomedical Centre, Uppsala, Sweden, respectively. Collagenase and Pronase were obtained from Sigma Chemical Co. and enterotoxin was purchased from P. E. Granum, Norwegian Food Research Institute, As, Norway. It was isolated from *Clostridium perfringens* as described previously (Granum & Whitaker, 1980). FHSA was prepared by the method of Mego *et al.* (1967). It was labelled with ^{125}I by the method of Redshaw & Lynch (1974).

Preparation of non-parenchymal liver cells

Liver cells were prepared from male Wistar rats (250g) by a collagenase perfusion technique (Tolleshaug *et al.*, 1977). Non-parenchymal cells were separated from the hepatocytes by differential centrifugation (Nilsson & Berg, 1977), or by incubation with Pronase (Berg & Boman, 1973) or enterotoxin (Berg *et al.*, 1979), treatments that selectively destroy the hepatocytes. In the studies *in vivo*, 0.1mg of ^{125}I-FHSA (5μCi) was injected intravenously into the right femoral vein 12min before the liver perfusion.

Centrifugal elutriation of non-parenchymal liver cells

A JE-6 elutriator rotor (Beckman Instruments, Palo Alto, CA, U.S.A.) equipped with a standard chamber was used in a J-21-type Beckman centrifuge. Non-parenchymal cells (20×10^6–100×10^6 cells) were introduced into the elutriation system in a Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]-buffered salt solution containing 1% bovine serum albumin (Tolleshaug *et al.*, 1977). Pronase-prepared non-parenchymal cells were centrifuged at 590*g* and enterotoxin-prepared non-parenchymal cells were centrifuged at 460*g*. The cells were introduced into the chamber at a flow rate of 11.3ml/min. Flow rate was then increased stepwise to 13.5, 20.3, 22.7, 25.4, 37.5, 53.0, 68.0 and 80.0ml/min, producing nine fractions, each with a volume of 100ml. The cells in the various fractions were concentrated by centrifugation at 1500*g* for 4min and then resuspended in a small volume of the elutriation fluid. About 80–90% of the cells applied to the elutriation system were recovered by this procedure.

Characterization of cells

Kupffer cells were identified cytochemically by positive peroxidase reaction (Wisse, 1974). Stellate cells were identified in a Leitz fluorescence microscope equipped with an A filter block (no. 513596). Cells with intense, but rapidly fading, fluorescence were taken to be stellate cells (Wake, 1980). Endothelial cells were identified in the fluorescence microscope with a K2 filter block (no. 513605), after incubation of the non-parenchymal liver cells at 37°C for 5h in the presence of 10μg of fluorescein amine-conjugated ovalbumin/ml. This compound is selectively taken up by the endothelial cells (Smedsrød *et al.*, 1982).

Formation of density gradients

Nycodenz gradients were prepared by the gradient-formation method of Stone (1974). Four solutions containing 27.5%, 18.3%, 13.8% and 5% Nycodenz were made by mixing the cell suspension with various amounts of 27.5% Nycodenz [27.5% (w/v) Nycodenz in 5mM-Tris/HCl, pH7.5, containing 3mM-KCl and 0.3mM-$CaNa_2EDTA$]. Portions (2.5ml) of these solutions were layered carefully on top of each other, and the tubes were turned to a horizontal position and left at 4°C for 90min. The tubes were subsequently turned upright, and the diffusion-generated gradients were centrifuged at 1300*g* for 60min. The gradients were fractionated by upward displacement with Maxidens.

Percoll gradients were prepared by mixing 7.5ml of Percoll solution [9 parts (v/v) of Percoll to 1 part (v/v) of 9% NaCl] with 5ml of cell suspension and centrifuged at 65000*g* for 60min. The gradients were fractionated by upward displacement with Maxidens.

Iron loading of Kupffer cells

Iron carbonyl (100mg in 200μl of 0.9% NaCl) was injected into the right femoral vein 30min before liver perfusion. The magnet (Eclipse, AX510; E. J. Neill and Co., Sheffield, U.K.) was covered with a sheet of Parafilm, and about 25ml of total cell suspension was poured on to the film (Berg & Boman, 1973). Cells that did not attach to the magnet were washed off into a beaker. The cells remaining on the magnet were washed with two 25ml portions of medium. The whole procedure was repeated twice. The cell suspensions were concentrated by centrifugation.

Isolation and cultivation of liver endothelial cells

Liver endothelial cells were isolated by centrifugal elutriation of enterotoxin-prepared non-parenchymal cells. Fractions 2–4 obtained by the elutriation process (see Fig. 1) were pooled, concentrated by centrifugation and resuspended in the incubation buffer (Tolleshaug *et al.*, 1977) or in the F-12 medium (2×10^6 cells/ml); 10^6 cells in F-12 medium were seeded in Linbro wells, which were coated with 10μg of fibronectin and allowed to adhere for 3h. The cells were then washed and incubated with different amounts of ^{125}I-FHSA in F-12 medium containing 20% (v/v) foetal-calf serum. Degradation (acid-soluble radioactivity) was determined by precipitation of the medium in 5% (w/v) trichloroacetic acid.

Results

Distribution of ^{125}I-FHSA in liver cells after injection intravenously

^{125}I-FHSA (0.1mg; 5μCi) was injected intravenously into the right femoral vein 12min before the liver perfusion. More than 80% of the injected radioactivity was in all experiments found in the liver, and more than 85% of the radioactivity in the liver was found in the non-parenchymal cells.

Distribution of ^{125}I-FHSA in non-parenchymal liver cells after centrifugal elutriation

Fig. 1 shows the distribution of endothelial cells, Kupffer cells and stellate cells, and the distribution of radioactivity in fractions after centrifugal elutriation of non-parenchymal cells prepared by the enterotoxin method. Most of the endothelial cells were recovered in the first three fractions. However, some endothelial cells aggregated, and were therefore washed out at a higher flow rate (fractions 6–8). The Kupffer cells peaked in fractions 6–8, and the stellate cells were mainly recovered in fraction 8. Most of the radioactivity was recovered in the first three fractions, but a second peak of radioactivity was observed in fractions 6–8. This result suggests that radioactive FHSA is mainly taken up by the endothelial cells in liver. The radioactivity recovered in fractions 6–8 may be associated either with the aggregated endothelial cells, with the Kupffer cells or with the stellate cells.

Fig. 2 shows results from an experiment in which non-parenchymal cells prepared by the Pronase method were elutriated. Endothelial cells and stellate cells were mainly found in fractions 2–4, whereas the Kupffer cells were mainly found in fraction 6. In this experiment we also gave 20i.u. of [^{3}H]retinol (90μCi) intraduodenally 18h before the liver perfusion. This compound is taken up by the stellate cells (Wake, 1980), and hence it may be used as a marker for these cells. No ^{125}I-labelled FHSA was found co-migrating with the Kupffer cells. The radioactive FHSA was recovered in the fraction containing both the endothelial cells and the marker for the stellate cells.

Taken together, the elutriation data indicate that ^{125}I-labelled FHSA is taken up *in vivo* mainly by the endothelial cells. Negligible amounts of radioactivity are recovered in the Kupffer cells. However, the possibility that some FHSA also is taken up by the stellate cells cannot be excluded from these data.

Distribution of ^{125}I-FHSA in non-parenchymal liver cells after density-gradient centrifugation

In these experiments, the Kupffer cells were removed from the initial liver-cell suspension by loading them with iron and subsequently removing them with a magnet. Up to 20% of the radioactivity found in liver was removed together with the Kupffer cells. This radioactivity most probably reflects the fact that some endothelial cells and stellate cells may adhere to the Kupffer cells, and are removed together with the Kupffer cells.

The rest of the non-parenchymal liver cells were then separated from the hepatocytes by differential centrifugation. This resulted in a cell suspension that consisted of endothelial cells and stellate cells. When endothelial cells and stellate cells prepared by this method were subjected to Nycodenz-

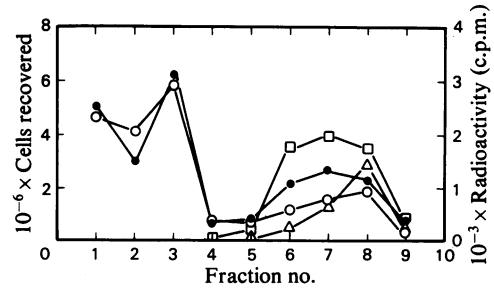

Fig. 1. *Distribution of ^{125}I-FHSA after centrifugal elutriation of non-parenchymal liver cells prepared by the enterotoxin method*

The Figure shows the distribution of Kupffer cells (□), endothelial cells (○) and stellate cells (△) and of ^{125}I-FHSA (●) in fractions obtained by centrifugal elutriation of non-parenchymal cells prepared by the enterotoxin method. ^{125}I-FHSA (0.1mg) was injected intravenously 12min before liver perfusion.

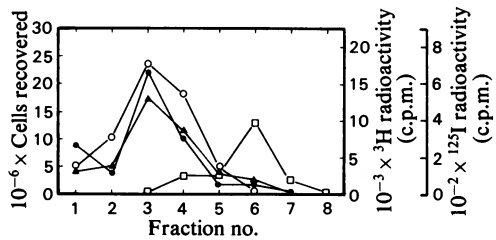

Fig. 2. *Distribution of ^{125}I-FHSA after centrifugal elutriation of non-parenchymal liver cells prepared by the Pronase method*

The Figure shows the distribution of peroxidase-positive cells (Kupffer cells) (□) and peroxidase-negative cells (endothelial cells and stellate cells) (○) and of [^{3}H]retinol (marker for the stellate cells) (▲) and ^{125}I-FHSA (●) in fractions prepared by centrifugal elutriation of non-parenchymal cells prepared by the Pronase method. Rats were given 20 i.u. of [^{3}H]retinol (90μCi) 18h before the cell preparation. ^{125}I-FHSA was injected intravenously 12min before liver perfusion.

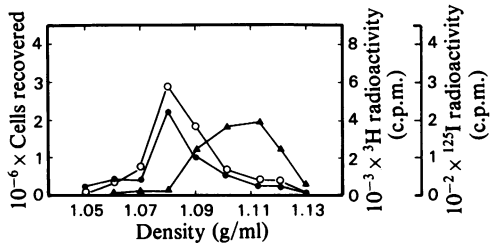

Fig. 3. *Distribution of ^{125}I-FHSA after Nycodenz-density-gradient centrifugation of endothelial cells and stellate cells*
The Figure shows the distribution of peroxidase-negative cells (endothelial cells and stellate cells) (○) and of [^{3}H]retinol (marker for the stellate cells) (▲) and ^{125}I-FHSA (●) after Nycodenz-density-gradient centrifugation. Non-parenchymal cells were prepared by differential centrifugation 18h after the rats had been given 20 i.u. of [^{3}H]retinol (90μCi). ^{125}I-FHSA was injected intravenously 12min before the liver perfusion. The Kupffer cells were removed from the suspension of non-parenchymal cells by loading the Kupffer cells with iron and subsequently removing these cells from the suspension with a magnet.

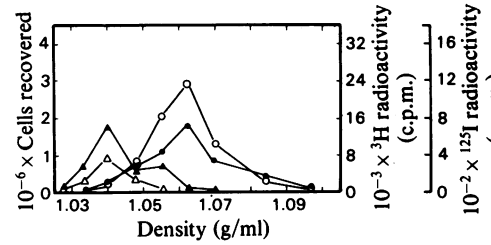

Fig. 4. *Distribution of ^{125}I-FHSA after Percoll-density-gradient centrifugation of endothelial cells and stellate cells*
The Figure shows the distribution of endothelial cells (○) and stellate cells (△) and of [^{3}H]retinol (▲) and ^{125}I-FHSA (●) in a Percoll density gradient. Non-parenchymal cells were prepared by differential centrifugation 18h after the rats had been given 20i.u. of [^{3}H]retinol (90μCi); 0.1mg of ^{125}I-FHSA (5μCi) was injected intravenously 12min before the liver perfusion. The iron-loaded Kupffer cells were removed from the cell suspension by the magnet method. Stellate cells and endothelial cells were identified by fluorescence microscopy.

density-gradient centrifugation, the stellate cells peaked at a density of about 1.11g/ml (shown by [^{3}H]retinol) and the endothelial cells peaked at a density of about 1.08g/ml (Fig. 3). When radioactive FHSA was injected intravenously 12min before liver perfusion, the radioactivity was found to co-migrate with the endothelial cells; very little ^{125}I-FHSA coincided with the stellate cells.

Fig. 4 shows results from an experiment in which iron-loaded Kupffer cells were removed from the cell suspension before density-gradient centrifugation in Percoll. Radioactive retinol and stellate cells peaked at a density of about 1.04g/ml. However, endothelial cells and ^{125}I-FHSA co-migrated and peaked at a density of about 1.06g/ml. Very little radioactivity from FHSA co-migrated with the stellate cells. These results indicate that ^{125}I-FHSA is taken up almost exclusively by the endothelial liver cells *in vivo*.

Degradation of ^{125}I-FHSA by liver endothelial cells in vitro

We have isolated endothelial cells from rat liver and determined the degradation of ^{125}I-FHSA by endothelial cells in suspension and in surface cultures. Cells in suspension as well as cells in monolayers took up and degraded ^{125}I-FHSA. The degradation started about 15min after uptake of the ligand. This lag phase most probably reflects the transport of the molecule from the plasma membrane to the lysosomes, where the degradation takes place (Wandel *et al.*, 1982). Fig. 5 shows the cellular degradation of radioactive

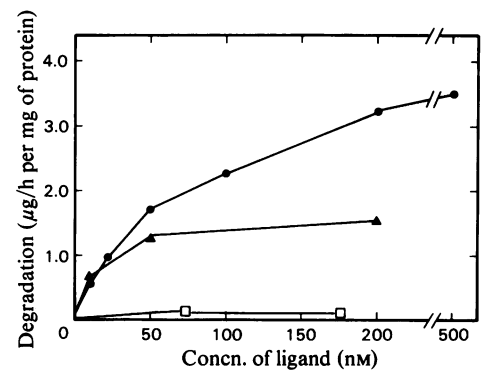

Fig. 5. *Degradation of ^{125}I-FHSA in endothelial cells in vitro*
Degradation of ^{125}I-FHSA by liver endothelial cells in suspension (2×10^{6} cells/ml) (▲) and in surface culture (●) was determined after 40min of incubation at 37°C. As a control, degradation of native ^{125}I-HSA was measured in a suspension of total non-parenchymal cells (5.7×10^{6} cells/ml) (□). The total non-parenchymal cells were prepared by differential centrifugation. Degradation (acid-soluble radioactivity) was determined by precipitation in 5% trichloroacetic acid.

FHSA after 40min of incubation as a function of the ligand concentration. At a concentration of 200nM, the rate of degradation of ^{125}I-labelled FHSA by the endothelial cells in suspension was about 1.7μg of ^{125}I-FHSA/h per mg of protein. After 7h in culture the rate of degradation had

increased to about 3·3 μg of ^{125}I-FHSA/h per mg of protein. As a control, we incubated a suspension of total non-parenchymal liver cells in the presence of different concentrations of native ^{125}I-labelled HSA. Insignificant degradation of native HSA was observed (Fig. 5).

Discussion

The present data demonstrate that FHSA is selectively taken up by liver endothelial cells after injection intravenously. The endothelial cells also maintained their ability to endocytose FHSA *in vitro*; cells in suspension as well as cells in surface culture took up and degraded ^{125}I-FHSA

Some preparations of ^{125}I-FHSA may to some extent be taken up by Kupffer cells after intravenous injection (10–20% of the injected dose) (results not shown). Uptake by Kupffer cells was observed when the denatured protein was stored for prolonged times (up to 8 months), and had formed aggregates. Such aggregates are probably taken up by Kupffer cells by phagocytosis (Buys *et al.*, 1975).

The critical modification induced by formaldehyde treatment of albumin is not known, although it is shown that it leads to increased negative charge of the molecule. A change in charge is also the result of acetylation and some other modifications of LDL and other ligands. The increased negative charge of the ligands may direct them to a common binding site, the scavenger receptor, which mediates their uptake and degradation.

Previous reports have shown that macrophages isolated from different organs exhibit scavenger receptors (Goldstein *et al.*, 1979; Fogelman *et al.*, 1980), and it was therefore suggested that the liver macrophages, the Kupffer cells, were responsible for the rapid clearance of denatured proteins after injection into mammals (Brown *et al.*, 1980). The present data show, however, that formaldehyde-denatured albumin is taken up by rat liver endothelial cells. This is in agreement with a preliminary report from Nagelkerke *et al.* (1982), who have shown that acetylated LDL is mainly taken up in liver by the endothelial cells.

Several ligands for the scavenger receptor may exist *in vivo*. One important ligand may be modified LDL. The liver is the main organ responsible for LDL catabolism (Pittman *et al.*, 1979), but endocytosis via the classical LDL receptor can only partly explain the uptake in liver. Receptor-mediated endocytosis via a receptor recognizing modified LDL may mediate uptake of considerable amounts of LDL. Such modified LDL may exist *in vivo*; interaction between blood platelets and LDL results in malonaldehyde-modified LDL (Haberland *et al.*, 1982), and aortic endothelial cells modify LDL in such a way that it is recognized by receptors in reticuloendothelial cells (Henriksen *et al.*, 1981). These modifications are most likely to be frequent at high LDL concentrations. A high LDL concentration is an important risk factor for atherosclerosis. Therefore the liver endothelial cells may be one major cell type involved in protection against atherogenic action of such lipoproteins after modification *in vivo*.

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