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Binding of acetylated low density lipoprotein and maleylated bovine serum albumin to the rat liver: one or two receptors?

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The liver is the major organ involved in clearance of acetylated low density lipoprotein (acetyl-LDL) and maleylated serum albumin (Mal-BSA). Quantitative analysis of the hepatic uptake by sequential scintigraphy in rats shows that the hepatic uptake capacity for Mal-BSA is at least 15 times larger than for acetyl-LDL particles. A membrane-associated M ~250 000 daltons hepatic receptor for acetyl-LDL and Mal-BSA was 1450-fold purified from total membrane by Triton X-114 solubilization, chromatography on polyethylenimine cellulose and gel filtration. This receptor incorporated into liposomes displayed a saturable binding of [^{131}I]Mal-BSA with a dissociation constant K_d = 15 nM and to [^{131}I]acetyl-LDL with a dissociation constant K_d = 0.9 nM. The binding of both ligands was sensitive to poly(vinyl sulfate). The purified scavenger receptor system has a binding capacity for [^{131}I]Mal-BSA 20 times larger than for [^{131}I]acetyl-LDL. This is similar to the maximal removal capacity of the rat liver for both ligands *in vivo*. Binding studies with Mal-BSA, acetyl-LDL and anti-idiotypic receptor antibodies as competitors for [^{131}I]Mal-BSA and [^{131}I]acetyl-LDL binding demonstrate that [^{131}I]Mal-BSA and [^{131}I]acetyl-LDL compete for a common binding site. However, not all of the Mal-BSA binding sites are capable of interacting with acetyl-LDL.

Key words: acetyl-LDL/Mal-BSA/scavenger receptor/anti-Mal-BSA anti-idiotypic antibodies

Introduction

The receptor for acetylated low density lipoprotein particles (acetyl-LDL), termed the 'scavenger receptor', was detected first on cultured murine peritoneal macrophages (Goldstein *et al.*, 1979). When these cells accumulate cholesterol ester via acetyl-LDL through the scavenger receptor pathway, they adopt a foamy appearance due to cytoplasmic lipid droplet formation. A potential role of the scavenger receptor in the pathogenesis of atherosclerosis has been suggested. Beside acetyl-LDL, the scavenger receptor binds LDL-like particles isolated from atherosclerotic aorta (Goldstein *et al.*, 1981) and oxidized LDL (Heinecke *et al.*, 1986). It was hypothesized that modified LDL would accumulate in monocytes/macrophages by the scavenger receptor causing the conversion of these cells into the foam cells, which are a typical cellular feature of the arterial wall in hypercholesterolemic state (Brown and Goldstein, 1983).

However, the true biological role of the scavenger receptor is still unclear. The scavenger receptor also recognizes maleylated bovine serum albumin (Mal-BSA) and some polyanionic compounds including poly(vinyl sulfate) (Brown *et al.*, 1980). *In vivo*, the predominant cellular site of scavenger receptor specific uptake and degradation of acetyl-LDL is the hepatic sinusoidal endothelial cell. This was demonstrated in rats after intravenous administration, and it was suggested that the hepatic scavenger receptor could provide a defense system against the accumulation of modified LDL particles in the blood (Nagelkerke *et al.*, 1983; Dresel *et al.*, 1985; Pitas *et al.*, 1985).

Previously a two-step procedure consisting of solubilization of total hepatic membranes with Triton X-114 and subsequent ion exchange chromatography on polyethylenimine cellulose in presence of β-D-octylglucoside was used to separate an acetyl-LDL receptor in an active form from the liver. The soluble receptor activity could be reconstituted into phospholipid and used for incubation with the radioligands and determination of the receptor-bound ligand by a membrane filtration assay. The receptor activity was visualized by ligand blotting of the active fractions from the PEI cellulose eluate. A predominant acetyl-LDL receptor species with an apparent mol. wt of 220 000–250 000 daltons was detected (Dresel *et al.*, 1985).

Recently Haberland *et al.* (1986) suggested that there exist two distinct receptors on human monocytes, one for Mal-BSA and the other for modified LDL. We have now examined this issue in the liver, the organ which expresses the most scavenger receptors *in vivo*.

Our study focuses on the following questions: are acetyl-LDL and Mal-BSA taken up by the liver in similar amounts? What are the binding characteristics of acetyl-LDL and Mal-BSA? Do they reflect the hepatic uptake of acetyl-LDL and Mal-BSA *in vivo*? Do acetyl-LDL and Mal-BSA compete quantitatively for a common binding site on the purified scavenger receptor?

Results

Uptake of [^{131}I]acetyl-LDL and [^{131}I]Mal-BSA by the rat liver

In this *in vivo* study, [^{131}I]acetyl-LDL and [^{131}I]Mal-BSA were administered to anesthesized rats by i.v. bolus injection to quan-

Table I. Hepatic uptake rates of an i.v. bolus of [^{131}I]acetyl-LDL and [^{131}I]Mal-BSA in rats

Radio-ligand	Rat	^{131}I-radio-ligand bolus (pmol/rat)	Hepatic uptake[a] (pmol/min × organ region)
Acetyl-LDL	1	200	37
	2	400	58
	3	2000	58
Mal-BSA	4	2205	162
	5	6615	626
	6	15 535	862

[a]Rates of the hepatic uptake were determined externally 1–3 min after injection of the i.v. bolus in single anesthesized rats by sequential scintiscans. The perfusion-corrected uptake data from the liver region are expressed as pmol radio ligand taken up by the liver region in a 1 min interval.

titate the liver capacity for uptake of two ligands of the hepatic scavenger receptor. The distribution of the radioactivity in the body of the animal was recorded subsequently by a sequential scintiscan and accumulation profiles obtained over different organ regions. When the amount of injected [^{131}I]acetyl-LDL was varied between 200 and 2000 pmol per rat, saturation of liver uptake was seen at 400 pmol acetyl-LDL. One to three minutes after the bolus injection the maximal hepatic uptake was 58 pmol/min × organ region (Table I). For [^{131}I]Mal-BSA, the hepatic uptake after i.v. bolus injections of 735−15 435 pmol [^{131}I]Mal-BSA was measured. It is apparent from the data shown in Table I, that the hepatic clearance capacity for [^{131}I]Mal-BSA is at least 15 times as high as that for [^{131}I]acetyl-LDL. This number is obtained with plasma concentrations of [^{131}I]Mal-BSA just below liver saturation levels. The maximal clearance capacity for [^{131}I]Mal-BSA is even higher than 862 pmol/min × liver.

To analyse whether [^{131}I]acetyl-LDL and [^{131}I]Mal-BSA compete for the scavenger receptor, a cross competition analysis was performed. When 2 mg of unlabelled Mal-BSA were injected 0.5 min before a 80 μg [^{131}I]acetyl-LDL bolus was administered, 82.8% of the hepatic uptake capacity for [^{131}I]acetyl-LDL was blocked between 1 and 10 min after the bolus injection. On the other hand, when 4 mg acetyl-LDL were pre-injected and followed by a bolus of 50 μg [^{131}I]Mal-BSA, only 15% of the liver uptake capacity for [^{131}I]Mal-BSA could be blocked. However, pre-injection of 2 mg poly(vinyl sulfate) inhibited very efficiently (>85%) the [^{131}I]acetyl-LDL and [^{131}I]Mal-BSA uptake by the liver. To clarify the molecular recognition mechanisms in the rat liver for [^{131}I]acetyl-LDL and [^{131}I]Mal-BSA, a biochemical analysis of the hepatic scavenger receptor was initiated.

Partial purification of the hepatic scavenger receptor

To find out whether the 220 000−250 000 daltons receptor species contains the putative scavenger receptor or possibly two receptors with different ligand specificities, the PEI-fraction was further purified to separate unspecific binding components in order to allow a precise differentiation of different classes of binding sites. To take advantage of the high mol. wt of the receptor species we used a gel filtration system for further purification, for analytical preparations a TSK G4000 h.p.l.c. column and for large scale preparation a Sephacryl S-500 column. Figure 1 shows the elution profile of a TSK G4000 column and the elution of a single peak with [^{131}I]Mal-BSA receptor activity and an apparent Stokes radius of 85 Å. Silver stains of polyacrylamide gels from various fractions demonstrate the effective separation of the receptor activity from the bulk of the solubilized membrane protein. By ligand blotting with acetyl-LDL, beside some possible aggregated material at the top of the gel, the 220 000−250 000 dalton receptor was visualized. Since the binding of acetyl-LDL was abolished in presence of poly(vinyl sulfate), this protein exhibits the specificity of the scavenger receptor.

For larger scale preparations of the scavenger receptor, up to 20 mg protein of the PEI fraction was applied to Sephacryl S-500 columns. The activity and purity of the fractions obtained by chromatography on this column was similar to the result of the

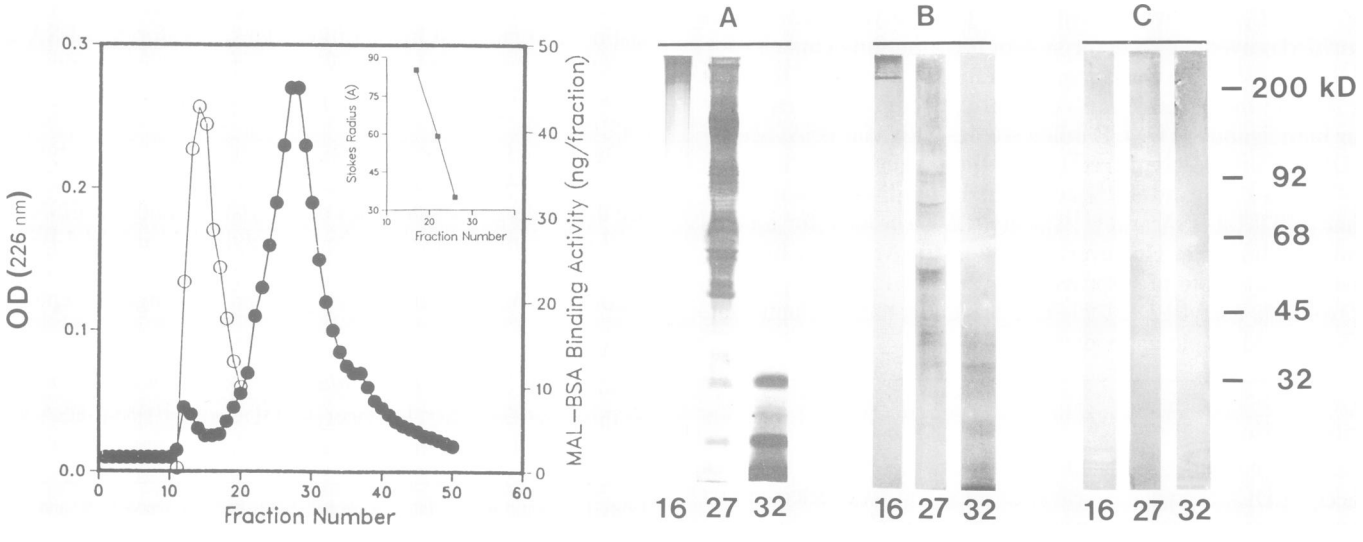

Fig. 1. Purification of [^{131}I]Mal-BSA binding sites by gel chromatography and visualization of the [^{131}I]acetyl-LDL binding site. The left panel shows the result of the final gel chromatogram: 200 μl of the previous PEI fraction (4 mg protein/ml) containing soluble acetyl-LDL receptor (Dresel *et al.*, 1985) were subjected to gel permeation chromatography on the TSK G4000 h.p.l.c. column (LKB, München) equilibrated in 50 mM Tris−Cl, pH 8, 50 mM NaCl, 2 mM EDTA and 0.1% SDS. The optical density was recorded continuously at 226 nm. The flow rate of the column was 1 ml/min at a constant pressure of 8 bars. Single fractions of 0.5 ml were collected. The void volume and the total volume of the column were determined in separated runs with blue dextran (Pharmacia, Freiburg) and potassium dichromate. The columns were calibrated with tyreoglobulin (85 Å), catalase (52 Å), albumin (35 Å), in presence and absence of 0.1% SDS. The distribution coefficients of the marker proteins in presence of 0.1% SDS were calculated from the ratio $(v_{protein} - v_{void})/(v_{total} - v_{void})$ and plotted against the known Stoke's radii (inset). The elution position of the scavenger receptor was determined by binding of [^{131}I]Mal-BSA by the filtration assay as described in Materials and methods. On the right, visualization of specific and unspecific binding of acetyl-LDL by binding sites from different fractions. The pool fractions no. 16−19, 27−30 and 32−35 from four TSK G4000 chromatograms were concentrated by centrifugation on Centricon 30 ultrafilter tubes (Amicon, Witten, FRG) and subjected in Laemmli-buffer to electrophoretic separation in 9% polyacrylamide slab gels containing 0.1% SDS. The lanes in group A show aliquots from the three pool/fractions after separation, stained by silver stain kit (Bio Rad, München, FRG) to visualize the proteins. The lanes in group B show aliquots after transblotting to nitrocellulose paper, incubation with acetyl-LDL (100 μg/ml) and subsequent anti-LDL-ELISA as described in Materials and methods. For visualization of the bound acetyl-LDL, the blots were developed in rabbit anti-human LDL IgG (20 μg/ml) and horse radish peroxidase conjugated goat anti-rabbit IgG, working dilution 1:667. As a control for non-specific binding, the lanes in group C containing identical aliquots to the group B lanes were incubated with acetyl-LDL (100 μg/ml) in presence of 50 μg/ml poly(vinyl sulfate) and further treated as the group B lanes.

experiment on TSK G 4000. Assuming that 100% of the scavenger receptor molecules in the S-500 pool could be reconstituted in a fully active state into egg phosphatidylcholine, the specific activity of the peak receptor fraction was up to 85 μg [^{131}I]acetyl-LDL/mg liposome-complexed protein. The binding of [^{131}I]acetyl-LDL to the liver membrane fraction was 58 ng/mg membrane protein (not shown). Thus an 1450-fold purification was achieved.

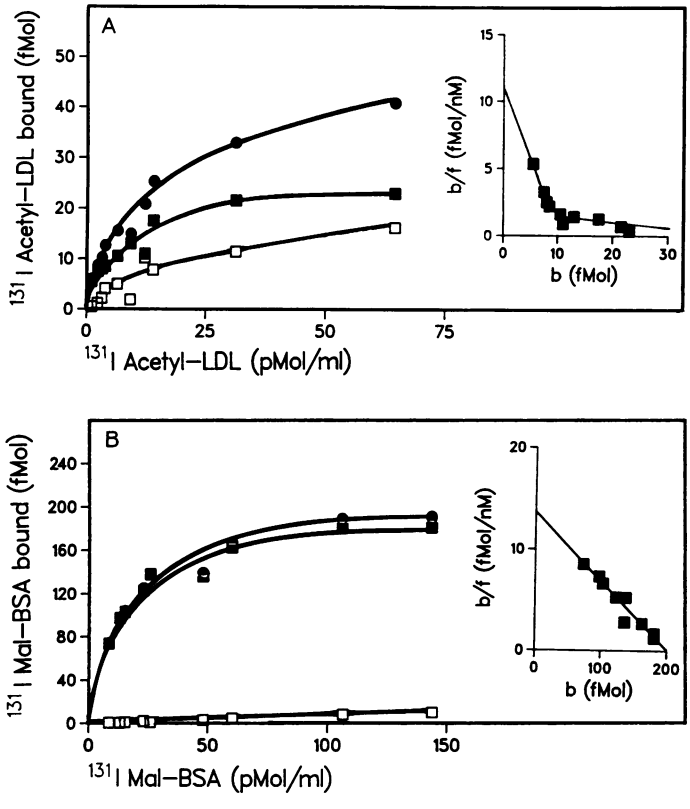

Fig. 2. Binding of [^{131}I]acetyl-LDL and [^{131}I]Mal-BSA to the highly purified hepatic scavenger receptor. Scavenger receptor purified by gel permeation chromatography was reconstituted with egg phosphatidylcholine as described in Material and methods. Aliquots of the scavenger receptor–liposome complexes (0.33 μg protein/assays) were incubated in TN-buffer + 2 mM EDTA and 1 mg/ml BSA with indicated concentrations of [^{131}I]acetyl-LDL (308 c.p.m./fmol) or [^{131}I]Mal-BSA (42 c.p.m./fmol) at room temperature for 30 min prior to filtration (to separate bound from free radio-ligand). The saturation binding profile of [^{131}I]acetyl-LDL is plotted on the upper left panel, the [^{131}I]Mal-BSA profile is on the left bottom panel. Scatchard analyses of binding data for the receptor-specific high-affinity binding curves are plotted on the right of each panel. ●, Total binding of the radio-ligands; ■, the receptor-dependent high-affinity binding; □, the non-receptor-dependent binding as determined in the presence of 40 μg/ml poly(vinyl sulfate). Values are the means from double determination.

Stoichiometry of [^{131}I]acetyl-LDL and [^{131}I]Mal-BSA binding to highly enriched scavenger receptor

To analyse the stoichiometry of acetyl-LDL binding and Mal-BSA binding to the fraction purified by gel chromatography, a saturation binding study with [^{131}I]acetyl-LDL, [^{131}I]Mal-BSA and receptor–liposome complexes was performed. For determining the receptor-specific binding capacity, the non-receptor-dependent binding was subtracted from the total binding. Then the maximal receptor specific binding component and the dissociation constant K_d could be determined by Scatchard analysis (Figure 2). The binding capacity of the scavenger receptor for [^{131}I]Mal-BSA binding per assay was 20-fold higher than for [^{131}I]acetyl-LDL when expressed by moles of bound radioligands (Figure 2). When transformed to Scatchard plots, the data show evidence for a single binding site for about 200 pmol [^{131}I]Mal-BSA/assay with an apparent K_d of 15 nM. For [^{131}I]acetyl-LDL, one high affinity binding site was detected which binds 10 pmol [^{131}I]acetyl-LDL/assay with an apparent K_d of 0.9 nM. A second type of binding site conforms to the characteristics expected for a low affinity site with a higher binding capacity (>30 pmol). Thus our highly enriched scavenger receptor fraction bound 20 times more [^{131}I]Mal-BSA molecules than [^{131}I]acetyl-LDL particles by a high affinity mechanism. The highly enriched scavenger receptor preparation has — comparable to the liver *in vivo* — a higher binding capacity for Mal-BSA. To compare the hepatic scavenger receptor activity of the rat with this receptor activity from other sources, murine P388 D.1 derived solid tumor tissue and bovine adrenal cortex were used for purification. Adrenal cortex is accumulating modified LDL very avidly (Pitas *et al.*, 1985). P388 D.1 derived tumor tissue, induced in syngeneic mice was previously demonstrated to have detergent-soluble scavenger receptor (Via *et al.*, 1985). The scavenger receptor containing fractions of the S-500 sepharose columns contained receptors with similar specific activities. The specific binding activities, B_{max}, K_d, and Mal-BSA/acetyl-LDL binding ratios are shown in Table II. Binding was also of high affinity (K_d = 1.6–1.7 nM) and sensitive to poly(vinyl sulfate). Saturation binding analysis with bovine adrenal cortex and murine P388 D.1 tumor tissue derived scavenger receptor S-500 fractions showed higher binding capacity for Mal-BSA than for acetyl-LDL at saturation conditions. The ratios of Mal-BSA/acetyl-LDL binding were 16.6:1 and 24:1. This suggests that scavenger receptor preparations not only from rat liver but also from other sources exhibit a similar and typical ratio of Mal-BSA/acetyl-LDL binding.

Specificity of binding sites in the highly enriched scavenger receptor

The specificity of the binding of [^{131}I]acetyl-LDL and [^{131}I]Mal-BSA was assayed by cross-competition assays in presence of

Table II. [^{131}I]Mal-BSA/[^{131}I]acetyl-LDL binding to Sephacryl S-500 fractions and dissociation constants

Source of Sephacryl S-500 fraction[a]	[^{131}I]Mal-BSA specific binding (pmol/μg)	K_d acetyl-LDL[b] (nM)	K_d Mal-BSA[b] (nM)	Maximal [^{131}I]Mal-BSA binding (pmol)[b] / Maximal [^{131}I]acetyl-LDL binding (pmol)
Rat liver membrane	0.6	0.9	15	20
Bovine adrenal membrane cortex	0.6	1.6	17.6	24
P388 murine macrophage-derived tumor tissue membrane	1.6	1.7	13.25	16.6

[a]Sephacryl S-500 fractions were purified from three different sources by the procedure described in detail in Material and methods. Specific binding activity for [^{131}I]Mal-BSA is expressed in pmol Mal-BSA bound per μg liposome-associated protein.
[b]B_{max} and K_d of [^{131}I]Mal-BSA binding and [^{131}I]acetyl-LDL binding were determined by filtration assay and Scatchard analysis of the binding data. The ratio of $B_{max\ Mal\text{-}BSA}/B_{max\ acetyl\text{-}LDL}$ was calculated from these data.

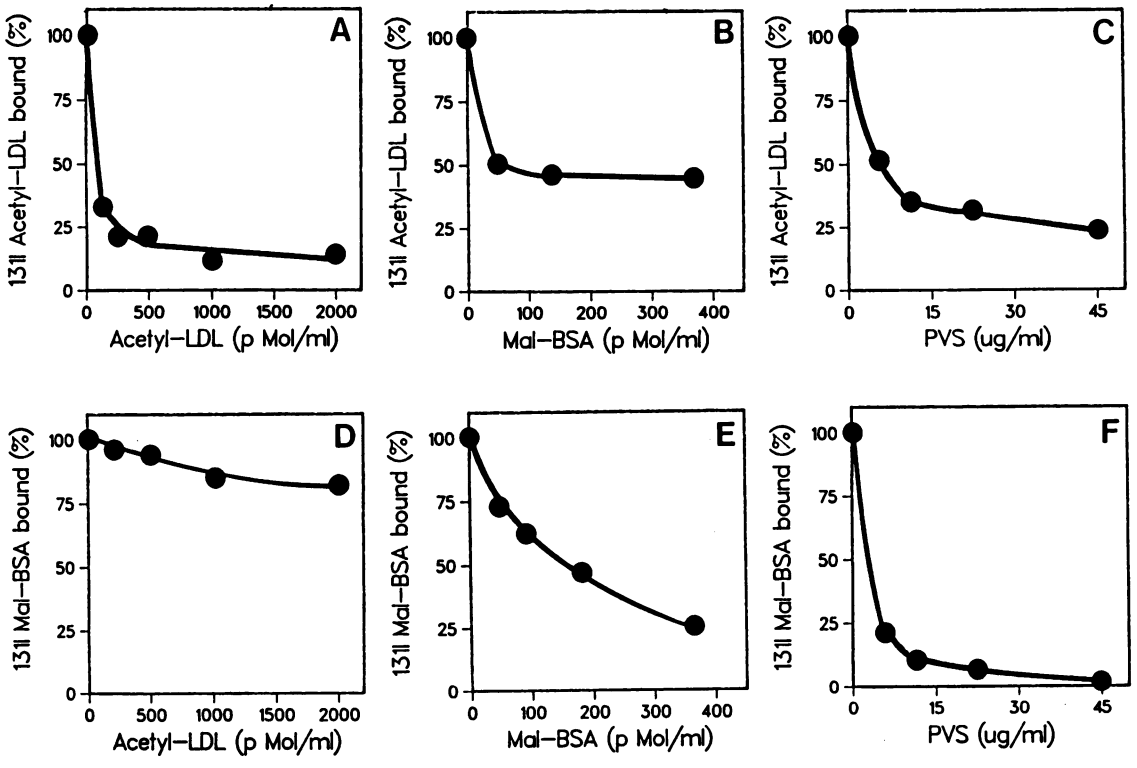

Fig. 3. Inhibition of [^{131}I]acetyl-LDL and [^{131}I]Mal-BSA to partially purified hepatic rat scavenger receptor by varying concentrations of unlabelled acetyl-LDL, Mal-BSA and poly(vinyl sulfate). Liposome–scavenger receptor complexes (1.9 μg protein/assay) from the PEI fraction were incubated with 20 pmol/ml [^{131}I]acetyl-LDL (700 c.p.m./fmol) or 23.6 pmol/ml [^{131}I]Mal-BSA (105 c.p.m./fmol) under the conditions described in the legend to Figure 2, in absence or presence of varying concentrations of unlabelled acetyl-LDL, Mal-BSA and poly(vinyl sulfate). **A–C** (upper panels) show the influence of excess of unlabelled acetyl-LDL **(A)**, unlabelled Mal-BSA **(B)** and poly(vinyl sulfate) on the total binding activity of [^{131}I]acetyl-LDL (100% = 42–44 fmol bound). **D–F** (bottom panels) show the influence of excess of unlabelled acetyl-LDL **(D)**, unlabelled Mal-BSA **(E)** and poly(vinyl sulfate) on the total binding activity of [^{131}I]Mal-BSA (100% = 450–515 fmol bound). Values are means from double determinations.

various concentrations of unlabelled ligands. In addition, the inhibition of the binding of the radioligands was studied in presence of poly(vinyl sulfate), to determine the level of 'non-receptor' dependent binding. The binding of [^{131}I]acetyl-LDL at a concentration of 20 pmol/ml decreased in presence of a 10- to 20-fold molar excess of unlabelled acetyl-LDL (Figure 3, upper panels) to the level of 'non-receptor' dependent binding. A 3- to 20-fold molar excess of Mal-BSA also dramatically reduced [^{131}I]acetyl-LDL binding. Double reciprocal plot analysis of [^{131}I]acetyl-LDL binding in the range of 1 pmol/ml to 65 pmol/ml in absence and presence of 735 pmol/ml unlabelled Mal-BSA indicated that Mal-BSA was a competitive inhibitor of the [^{131}I]acetyl-LDL binding (Figure 4, upper panels).

To examine competition of [^{131}I]Mal-BSA binding, inhibition studies were performed at [^{131}I]Mal-BSA concentrations of 23.5 pmol/ml [^{131}I]Mal-BSA (Figure 3, lower panels). The figures show a drastic inhibition of [^{131}I]Mal-BSA binding in presence of an up to 16-fold excess of unlabelled Mal-BSA decreasing to non-receptor binding. However, even a 45-fold excess of unlabelled acetyl-LDL did not compete for more than 19% of the total binding capacity for [^{131}I]Mal-BSA. A double reciprocal plot of the [^{131}I]Mal-BSA saturation binding data in the range of 9.2 pmol/ml to 130 pmol/ml in absence and presence of 2000 pmol/ml acetyl-LDL shows that acetyl-LDL is a comeptitive inhibitor for the Mal-BSA binding sites (Figure 4). Thus, competition studies and the double reciprocal plot analysis of the binding data provide evidence that acetyl-LDL and Mal-BSA bind to common binding sites. However, based on the inhibition with acetyl-LDL we suggest that the Mal-BSA binding sites can be grouped into two classes — those which can be competed by acetyl-LDL and those which are unaffected by acetyl-LDL. Poly(vinyl sulfate) is an effective antagonist for both groups of Mal-BSA binding sites.

Competition of [^{131}I]acetyl-LDL and [^{131}I]Mal-BSA binding to the scavenger receptor by anti-Mal-BSA anti-idiotypic antibodies

In an attempt to differentiate the Mal-BSA binding sites immunologically, we used anti-Mal-BSA anti-idiotypic antibodies. Anti-Mal-BSA antibodies were used to generate anti-Mal-BSA anti-idiotypic antibodies. Such antibodies would also interact with the binding site of the scavenger receptor and thus act as a competitive antagonist like Mal-BSA. Anti-idiotypic antibodies had been successfully developed against other surface membrane receptors (Sege and Peterson, 1978; Homcy *et al.*, 1982). Anti-idiotypic antibodies against rabbit anti-Mal-BSA IgG were obtained by immunizing rats. The total IgG fraction was used for immunization in order to avoid leakage of the original hapten. Anti-anti-Mal-BSA IgG was isolated by ammonium sulfate precipitation and chromatography on DEAE cellulose from immune serum. To show anti-idiotypic antibody activity, we tested the binding of [^{131}I]acetyl-LDL to receptor–liposome complexes in presence of varying concentrations of immune-IgG. For control experiments rat anti-rabbit pre-immune IgG was used (Figure 5). By increasing concentrations of anti-Mal-BSA antibodies the binding of [^{131}]acetyl-LDL was inhibited to the same extent by anti-Mal-BSA antibodies as with an excess of unlabelled Mal-BSA (Figures 3 and 5). The control experiment did not show a significant inhibition of [^{131}I]acetyl-LDL binding to the recep-

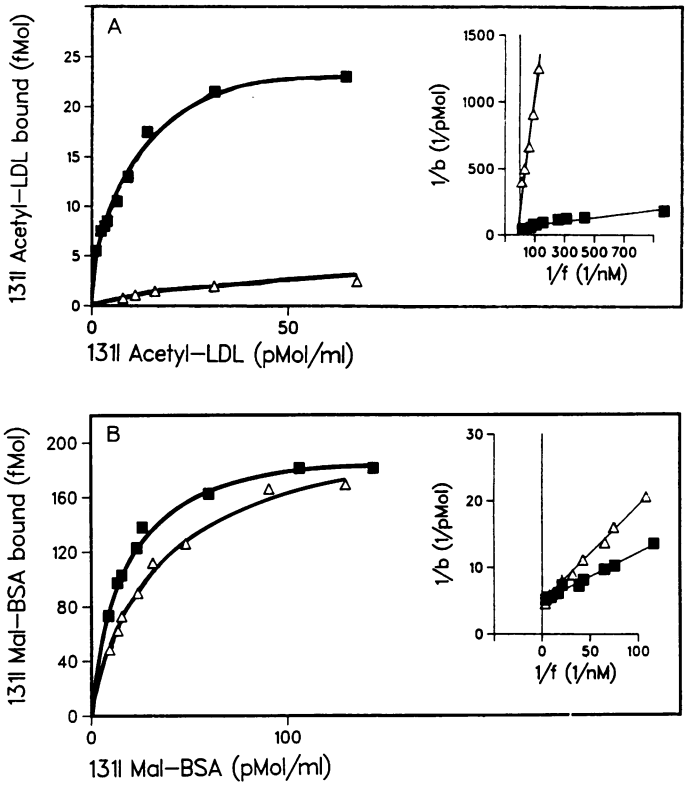

Fig. 4. Determination of scavenger receptor specific binding of [^{131}I]acetyl-LDL in absence and presence of an excess Mal-BSA and receptor-specific binding of [^{131}I]Mal-BSA in absence and presence of an excess of acetyl-LDL (saturation analysis of the scavenger receptor in presence of a second binding protein). Liposome–scavenger receptor complexes (0.33 μg protein/assay) were obtained and incubated with indicated concentrations of [^{131}I]radio-ligands as described in the legend to Figure 2, in the absence and presence of an excess of the second, unlabelled ligand. The upper panel **(A)** shows at the left the saturation binding curve for the receptor-dependent binding of [^{131}I]acetyl-LDL (308 c.p.m./fmol). ■, Receptor-dependent binding in absence of Mal-BSA; ▵, receptor-dependent binding in presence of 735 pmol/ml Mal-BSA. The data of double reciprocal analysis for the receptor data are plotted at the right. The bottom panel **(B)** shows at the left the saturation binding curve and double reciprocal analysis for the receptor-dependent binding of [^{131}I]Mal-BSA (42 c.p.m./fmol). ■, Receptor-dependent binding in absence of acetyl-LDL; Δ, receptor-dependent binding in presence of 2000 pmol/ml acetyl-LDL. Values are the means from double determinations.

tor–liposome complexes. This result indicates that anti-Mal-BSA anti-idiotypic antibodies are present, possibly competing for the binding sites of the scavenger receptor. To show that anti-idiotypic antibodies are directed against the specific ligand recognition site of the scavenger receptor, saturation binding of acetyl-LDL and Mal-BSA was examined in absence and presence of anti-Mal-BSA anti-idiotypic antibodies. Saturation binding analysis and double reciprocal analysis of the saturation binding data of [^{131}I]acetyl-LDL and [^{131}I]Mal-BSA in absence and presence of anti-Mal-BSA anti-idiotypic antibodies show that these antibodies act as competitors for the binding of [^{131}I]acetyl-LDL and [^{131}I]Mal-BSA, since they affect the slope of the linear intercept of the *y*-axis of the reciprocal plot as expected for a competitive inhibitor (Figure 6).

Binding of the scavenger receptor fraction to immobilized acetyl-LDL

In an attempt to separate the two groups of Mal-BSA binding sites biochemically, acetyl-LDL particles were immobilized on Sepharose 4B to be used for affinity chromatography. Our hope

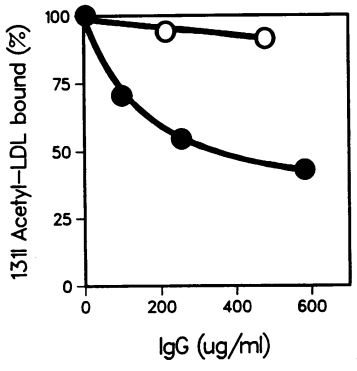

Fig. 5. Inhibition of the binding of [^{131}I]acetyl-LDL by anti-Mal-BSA anti-idiotypic antibodies. Liposome–scavenger receptor complexes (1.9 μg protein/assay) from the PEI fraction were incubated with 20 pmol/ml [^{131}I]acetyl-LDL and varying concentrations of purified rat anti-rabbit anti-Mal-BSA IgG to demonstrate anti-idiotypic antibodies against anti-Mal-BSA IgG (●) and with rat anti-rabbit anti IgG as a control (○).

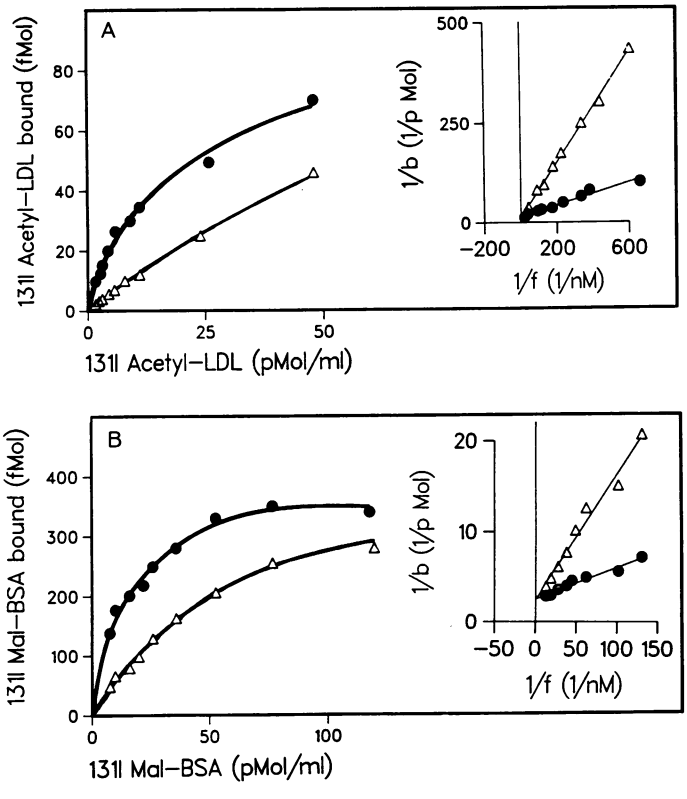

Fig. 6. Competitive inhibition of [^{131}I]acetyl-LDL and [^{131}I]Mal-BSA binding to partially purified hepatic scavenger receptor by anti-Mal-BSA anti-idiotypic antibodies. Liposome–scavenger receptor complexes (0.33 μg protein/assay) were obtained and incubated under conditions described in the legend to Figure 2, with indicated concentrations of [^{131}I]radio-ligands in absence and presence of anti-Mal-BSA-anti-idiotypic antibodies (125 μg immune-IgG per assay). The upper panel **(A)** shows at the left the saturation binding curve for the receptor dependent binding of [^{131}I]acetyl-LDL (589 c.p.m./fmol): ●, binding in absence of IgG; Δ, binding in presence of anti-Mal-BSA anti-idiotypic antibodies. The data of double reciprocal analysis for the binding data are plotted at the right. The bottom panel **(B)** shows at the left the saturation binding curve for binding of [^{131}I]Mal-BSA (97 c.p.m./fmol): ●, binding in absence of IgG; Δ, binding in presence of anti-Mal-BSA anti-idiotypic antibodies. On the right again the double reciprocal analysis of the binding data.

was that the acetyl-LDL Sepharose would retain the scavenger receptor but not — if located on a second molecular species — a second Mal-BSA binding site which could not be competed

Table III. Binding of the scavenger receptor activity to immobilized acetyl-LDL[a]

Fraction	[^{131}I]Mal-BSA binding capacity (μg)	[^{131}I]Mal-BSA/[^{131}I]acetyl-LDL binding ratio
Applied	3–6	20
Recovered (flow through)	0.9–1.9	18.5

[a]Up to 8 μg [^{131}I]Mal-BSA binding activity were prepared from rat liver from the 100 000 *g* pellet by chromatography on PEI-cellulose and Sephacryl S-500. After removal of the detergent up to 6 μg [^{131}I]Mal-BSA binding activity were passed over an acetyl-LDL Sepharose column (1 cm^3) containing 12 mg immobilized acetyl-LDL. The binding capacity of the fractions applied to the column and recovered in the flow through was reconstituted into liposomes and quantified as described in Material and methods.

with an excess of acetyl-LDL. Since detergents prevent the binding of the scavenger receptor to acetyl-LDL (not shown), the receptor fractions had to be passed over a Sephadex G25 column to remove the detergent. After removal of the detergent 80% of the receptor activity could be recovered and was immediately applied to acetyl-LDL Sepharose. In four experiments we found that this column retains more than 65% of the applied [^{131}I]Mal-BSA binding activity. Moreover the ratios of [^{131}I]Mal-BSA/[^{131}I]acetyl-LDL binding in the flow throughs were not affected (Table III). Therefore we were unable to obtain evidence for two Mal-BSA binding sites on two receptor molecules which could be separated on a specific affinity column.

Discussion

We have used a combined approach of *in vivo* techniques and biochemical and immunological methods to address the question of binding of acetyl-LDL and Mal-BSA to the hepatic scavenger receptor. Quantitative analysis of [^{131}I]acetyl-LDL and [^{131}I]Mal-BSA uptake by the liver after intravenous bolus injection calculated from gamma camera records over the liver region clearly demonstrates that the hepatic uptake capacity for [^{131}I]Mal-BSA is at least 15 times larger than for [^{131}I]acetyl-LDL particles. Likewise, highly purified receptor from liver, adrenal and P388 D.1 murine tumors showed a distinction between the amount of radio-labelled Mal-BSA and acetyl-LDL bound. For example, the partially purified hepatic scavenger receptor fraction had a 20-fold higher binding capacity for Mal-BSA than for acetyl-LDL. Scatchard analysis of the saturation binding data revealed the presence of a single class of high affinity binding sites for each ligand: a single class of high affinity binding sites for [^{131}I]acetyl-LDL with a dissociation constant (K_d) of 0.9 nM and a binding capacity of 30 pmol/μg protein and a single class of high affinity binding sites for [^{131}I]Mal-BSA with a dissociation constant (K_d) of 15 nM and 600 pmol/μg protein binding capacity. When the highly purified scavenger receptors were saturated, they reflect the characteristic uptake difference between acetyl-LDL and Mal-BSA *in vivo* by the liver. The comparable scavenger receptor fractions isolated from different tissues out of different species show also a 16.6:1–24:1 binding ratio for Mal-BSA/acetyl-LDL at saturation.

Previous inhibition studies of cellular binding and uptake of acetyl-LDL have shown that Mal-BSA and anionic poly(vinyl sulfate) drastically reduce the binding of iodinated acetyl-LDL to the scavenger receptor of cultured murine peritoneal macrophages (Brown *et al.*, 1980). Our inhibition studies of the [^{131}I]acetyl-LDL binding in presence of Mal-BSA and poly(vinyl sulfate) with highly enriched hepatic scavenger receptor fractions confirm these observations and demonstrate a competitive inhibition.

We also studied the inhibition of [^{131}I]Mal-BSA binding by poly(vinyl sulfate) and excess of acetyl-LDL. Our data indicate that poly(vinyl sulfate) is a potent inhibitor for Mal-BSA binding sites as well, but acetyl-LDL apparently is not. It competed only for 20% of the Mal-BSA binding sites. Results from *in vivo* studies support this observation, since pre-injection of acetyl-LDL antagonized only about 15% of the hepatic Mal-BSA uptake. Even after the scavenger receptor activity has been purified 1450-fold, the Mal-BSA and acetyl-LDL binding reveals this stereospecificity, indicating that the receptor sites remain intact.

Some Mal-BSA binding sites differ in their recognition of acetyl-LDL. Therefore one might consider grouping the Mal-BSA binding sites into two subclasses. This, however, raises the question whether the two subclasses of Mal-BSA binding sites present evidence for two receptors, a separate Mal-BSA receptor and the scavenger receptor with its binding site for acetyl-LDL or Mal-BSA. Besides the linearity of Scatchard plots in the high affinity range, several lines of evidence argue against this possibility. First, when the partially purified scavenger receptor fraction was passed over an acetyl-LDL Sepharose column, the acetyl-LDL binding activity and Mal-BSA binding activity were effectively retained. Second, poly(vinyl sulfate) inhibits both Mal-BSA subclasses at identical concentrations. Third, anti-Mal-BSA anti-idiotypic antibodies competed for both subclasses with the same efficiency and did not differentiate the two subclasses immunologically. Fourth, regardless of the total number of receptor sites isolated from three different sources, the ratio of Mal-BSA binding to acetyl-LDL binding seems to be in a similar range.

Haberland *et al.* (1986) reported evidence for two distinct Mal-BSA receptors on freshly isolated human monocytes, the scavenger receptor and a Mal-BSA receptor. However, high concentrations of Mal-BSA were required in their experiments to demonstrate a second uptake process. Maximal binding to this site occurs at 7–10 μM, a concentration far greater than that required for saturation of the scavenger receptor. The binding of Mal-BSA to this binding site of the human monocyte is not sensitive to the known competitors of the scavenger receptor. At the much lower ligand concentrations, and the plasma concentrations *in vivo*, we did not see evidence that this binding determinant constitutes a quantitatively important number of additional binding sites beside the scavenger receptor in the rat liver.

The most likely model of the scavenger receptor on the basis of our data is a complex high mol. wt structure with multiple binding domains or tightly linked subunits containing binding sites. The most feasible explanation for the lower number of binding sites for acetyl-LDL particles as compared to Mal-BSA molecules at maximal receptor occupancy, and only partial inhibition of Mal-BSA binding by excessive acetyl-LDL particles is that the scavenger receptor is in a conformation that allows the essential groups of all binding sites to interact with Mal-BSA but not with the much larger acetyl-LDL particles. The isolation of a functional subunit and identification of binding domains is now in progress.

Finally, the fact that anti-Mal-BSA anti-idiotypic antibodies could be raised should make it possible to produce monoclonal anti-idiotypic anti-scavenger receptor antibodies. Such antibodies help to identify the still unknwon ligands of the scavenger receptor *in vivo*.

Materials and methods

Materials

Sodium[^{131}I]iodide (10–20 mCi/μg) was obtained from Amersham; egg phosphatidylcholine and Triton X-114 from Serva, Heidelberg; octyl-β-D-glucoside from Sigma; Sephacryl S-1000 Superfine and cyanogen-bromide-activated Sepharose 4B from Pharmacia (Uppsala/Sweden); TSK G4000 column (length 30 cm) from LKB. Poly(vinyl sulfate) was from Sigma, St Louis, MO, USA; cellulose acetate membrane filters (diameter 25 mm, pore size. 45 μm, cat. no. HULP 025 00) were from Millipore Corp., Bedford, MA 01730, USA.

Lipoproteins, maleylated serum albumin, iodination

Human LDL (density, 1.019–1.063 g/ml) was prepared from plasma of normal blood donors by sequential ultracentrifugation (Havel *et al.*, 1955). Acetyl-LDL was prepared by treatment of LDL with acetic anhydride (Basu *et al.*, 1976). Lipoprotein concentration was expressed in terms of protein content. BSA was maleylated with maleic anhydride as described (Imber *et al.*, 1982). Assays with 2,4,6-trinitrobenzenesulfonic acid with native and maleylated albumin by published procedures (Fields, 1972) indicated that 50 out of 59 lysine of the albumin were maleylated.

The radio-iodination of acetyl-LDL and Mal-BSA with Na[^{131}I] was performed under optimized conditions by the use of *N*-bromo-succinimide (NBS) as oxidizing agent to generate $^{131}I^+$ as described by us elsewhere (Sinn *et al.*, in preparation). Our typical iodination mixtures (0.5 ml) contained 500–1000 μg protein or lipoprotein, 0.4–1.6 mCi Na[^{131}I] (0.04–0.16 μg), and up to 3 μg NBS. These assays were incubated for 2 min at room temperature before termination of the iodination by dilution with 0.25 M phosphate buffer. Free iodine was removed by centrifugation on Centricon 30 ultrafilter and subsequent overnight dialysis against 10 mM Tris, pH 8, 150 mM NaCl, 0.1 mM EDTA.

Immunization protocol, antibody purification

Rabbits were immunized with 0.5–1 mg of the maleylated albumin in complete Freud's adjuvant by multiple site subcutaneous injection at 2 week intervals. High titer binding was detected after 6 weeks of immunication. Immunoglobulin G was precipitated from plasma by 40% ammonium sulfate and subsequently dialysed into 10 mM phosphate buffer, pH 7.4. Then the proteins were passed over a DEAE column equilibrated in the same buffer. The IgG was in the flow-through and inhibited [^{131}I]Mal-BSA binding to partially purified scavenger receptor.

To generate an anti-idiotypic response, rats were immunized with rabbit-anti-Mal-BSA IgG in complete Freund's adjuvant with 1–2 mg IgG subcutaneously at the back at 2 week intervals. After 16 days, animals were exsanguinated. IgG was purified by 40% ammonium sulfate precipitation and DEAE chromatography in phosphate buffer, pH 7.4. The immunoglobulin was detected in the flow through. SDS–PAA gel electrophoresis under non-reducing conditions demonstrated a single band at 150 000 daltons by silver staining. Following the same protocol, rat anti-rabbit IgG was prepared for control assays.

Assays for binding of [^{131}I]acetyl-LDL and [^{131}I]Mal-BSA

Binding to the soluble membrane proteins eluted from the polyethylenimine cellulose column, TSK G4000 column, and Sephacryl S-500 column, was determined by a filtration assay. Aliquots of the eluted proteins were diluted to submicellar detergent concentration, complexed with egg phosphatidylcholine and precipitated by acetone as described previously (Via *et al.*, 1985). After resuspension, the liposome–protein complexes were subsequently incubated with [^{131}I]acetyl-LDL, [^{131}I]Mal-BSA in absence and presence of unlabelled inhibitors or immunoglobulin. Unbound from bound radio-ligands were separated by membrane filtration on cellulose acetate filters (Schneider *et al.*, 1980). Non-receptor-dependent binding of [^{131}I]acetyl-LDL and [^{131}I]Mal-BSA was obtained in presence of 40 μg/ml of poly(vinyl sulfate). Receptor-dependent binding was calculated by subtracting the value for non-receptor depending binding of the radio-ligands from the value of total binding. The data for receptor-specific binding were used for construction of linear plots of the ratio of receptor-bound radio-ligand (Scatchard, 1949). The number of receptor-bound ligands at saturation, B_{max}, was given by the X-intercept of the linear plot. The equilibrium dissociation constant, K_d, was given by the ratio of B_{max} and the Y-intercept.

Solubilization and fractionation of membrane proteins

The procedure was described by us most recently (Dresel *et al.*, 1985). Briefly, the liver was disrupted in TNE buffer (50 mM Tris–Cl, 150 mM NaCl, 0.1 mM EDTA; 4 ml buffer/mg tissue) and centrifuged for 10 min at 3000 *g*. Then the supernatant was centrifuged for 60 min at 100 000 *g*. The pellet was dissolved in TNE buffer + 1% Triton X-114 at 4°C and clarified by centrifugation for 30 min at 100 000 *g*. Then this fraction was warmed up to 30°C and the hydrophobic and amphiphilic phases were separated by phase separation (Bordier, 1981). The amphiphilic phase was applied to polyethylenimine cellulose columns (100 mg protein/10 ml column volume). Triton X-114 was replaced by 40 mM *n*-octyl-β-D-glucopyranoside. The soluble membrane proteins containing the acetyl-LDL binding activity were eluted by a salt step (1500 mM NaCl) in TNE buffer + 40 mM *n*-octyl-β-D-glucopyranoside and assayed by filter assay or by ligand blotting as described by us (Dresel *et al.*, 1984; Via *et al.*, 1985; Dresel *et al.*, 1985). P388 D.1 derived tumor tissue was produced as described (Via *et al.*, 1985). Bovine adrenal cortex was obtained after slaughter. Both tissues were fractionated and for scavenger receptor purification like rat liver.

Gel electrophoresis

SDS–polyacrylamide gel electrophoresis was performed by the procedure of Laemmli (Laemmli, 1970) on slab gels. Gels were calibrated with high mol. wt standard proteins in the mol. wt range of 40 000–250 000 daltons obtained from Bio-Rad (Bio-Rad, Richmond, CA, USA, cat. no 161–0303). Proteins were stained with the commercial Silver Stain from Bio-Rad.

Ligand blotting

Proteins separated by polyacrylamide gels (Laemmli, 1970) were transferred to nitrocellulose paper (Towbin *et al.*, 1979) and incubated with a saturated solution of casein in TNE buffer for 2 h. To visualize acetyl-LDL binding proteins the strips were incubated with acetyl-LDL in absence and presence of poly(vinyl sulfate) as described (Dresel *et al.*, 1985). The assay was developed by sequential incubation with rabbit anti-human LDL IgG (5 μg/ml) and goat anti-rabbit IgG horse-radish peroxidase conjugate (1:667) for 60 min at room temperature in presence of 0.018% 4-chloro-2-naphthol and 10 μl H_2O_2/50 ml vol (Dresel *et al.*, 1984). Radio-ligand blots with [^{131}I]Mal-BSA were obtained under identical incubation conditions and autoradiographed on Kodak XR-5 films.

Scintillation camera imaging of the hepatic scavenger receptor

The gamma camera device, the software, and the experimental conditions for continuous scintigraphy of the hepatic scavenger receptor in rats was described by us earlier (Dresel *et al.*, 1985). [^{131}I]acetyl-LDL (2000–5000 c.p.m./pmol) and [^{131}I]Mal-BSA (2000–3000 c.p.m./pmol) were injected in the tail vein of anesthesized rats. Data recording was started 0.5 min before injection. Time activity curves were recorded for total body radioactivity and over the liver region.

Gel permeation chromatography

For small scale chromatography we used TSK gel G4000 SW performed columns 300 mm in length, with a diameter of 7.5 mm from LKB (S-16126 Bromma, Sweden, cat. no. 2135-430). Before application of the PEI fraction proteins OG was exchanged for 0.1% SDS by chromatography on Sephadex G25 pre-equilibrated in 50 mM Tris–Cl, pH 6.8, 0.1 mM EDTA, 50 mM NaCl, 0.1% SDS. Then the samples were passed through 0.45 μm millipore filters (Nikon Millipore Kogyo K.K., Yonezawa, Japan, cat no. SJHVL 04 NS). The typical sample size for a TSK G4000 SW column pre-equilibrated in 50 mM Tris–Cl, pH 8, 1 mM EDTA, 0.1% SDS was 200 μl containing 1.5 mg/ml protein. Chromatography was performed at a flow rate of 0.5 ml/min with constant pressure. For calibration of the column standard proteins with known Stoke's radii were chromatographed.

Gel permeation chromatography on a larger scale was performed on Sephacryl S-500. The column length was 900 mm, the diameter was 25 mm. Aliquots (2.5 ml) of the PEI fraction were applied and fractions of 12 ml were collected. The flow rate was 72 ml/h.

Chromatography of partially purified scavenger receptor on acetyl-LDL Sepharose 4 B

Acetyl-LDL-Sepharose 4 B was prepared by attaching LDL covalently to cyanogen-bromide-activated Sepharose 4 B according to Schneider *et al.* (1982). Using a ratio of 30 mg LDL-protein per g dry gel, 26.5 mg LDL could be bound to the activated Sepharose 4 B. The acetyl-LDL Sepharose was acetylated by the procedure of Basu *et al.* (1976) and equilibrated in 50 mM Tris–HCl (pH 8), 150 mM NaCl, 1 mM EDTA (TNE-buffer), supplemented with 0.02% (w/v) NaN_3. For affinity chromatography 10–20 ml aliquots (3–8 μg [^{131}I]Mal-BSA binding capacity) of a Sephacryl S-500 fraction were subjected to a Sephadex G 25 column (size 30 × 2.5 cm) equilibrated in TNE-buffer. In the void volume 85% of the receptor activity was detected. Before application to the acetyl-LDL Sepharose 4 B this fraction was centrifuged for 10 min at 60 000 *g*. Ninety percent of the receptor was in the supernatant and immediately passed three times over an acetyl-LDL Sepharose 4 B column (1 cm^3) with a flow rate of 0.1 ml/min. The flow-through was collected for assaying the [^{131}I]Mal-BSA/[^{131}I]acetyl-LDL binding ratio.

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