Purification of low density lipoprotein receptor from liver and its quantification by anti-receptor monoclonal antibodies

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The low density lipoprotein (LDL) receptor has been purified to homogeneity from rabbit liver by a combination of DEAE-Sephacel chromatography, LDL-Sepharose 4B chromatography and preparative SDS/polyacrylamide-gel electrophoresis. The receptor protein had a *pI* of 4.45 and an *M_r* of 120×10^3 – 125×10^3 in SDS gels under non-reducing conditions. Incubation of the LDL receptor with neuraminidase decreased its *M_r* to 105×10^3 – 110×10^3 and increased its *pI* from 4.45 to 5.25. The purified receptor exhibited all the properties of the membrane-bound receptor including Ca^{2+} -dependent binding of rabbit and human LDL but not of methylated LDL or high density lipoprotein. The amount of LDL receptor present in rabbit liver was measured by a quantitative blotting procedure employing a newly developed rat anti-receptor monoclonal antibody. The affinity and specificity of this monoclonal antibody allowed the quantification of the LDL receptor in detergent extracts of liver homogenate, thus eliminating the loss of receptor associated with the preparation of membrane fractions prior to receptor assay. Livers from adult female New Zealand White rabbits contained 149 ± 13 ng of LDL receptor/mg of liver protein. Administration of pharmacological doses of 17α -ethinyloestradiol raised the concentration of LDL receptor in liver to 312 ± 25 ng/mg of liver protein.

INTRODUCTION

It is now well established from clearance studies carried out *in vivo* that the majority of serum LDL is catabolized through the LDL receptor pathway (Pittman *et al.*, 1979; Mahley *et al.*, 1980; Slater *et al.*, 1980; Pittman *et al.*, 1982; Carew *et al.*, 1982; Kesaniemi *et al.*, 1983; Steinbrecher *et al.*, 1983; Spady *et al.*, 1983). Studies in several species, including man, have shown that this process occurs predominantly in the liver (Pittman *et al.*, 1979; Slater *et al.*, 1980; Carew *et al.*, 1982; Pittman *et al.*, 1982; Spady *et al.*, 1983; Starzl *et al.*, 1984).

Several lines of evidence suggest that the activity of the LDL receptor in liver is important in controlling the level of circulating LDL and, therefore, much work is now being directed to study the factors and mechanisms which regulate the expression of the receptor in this tissue. Such studies, however, require suitable assays for the receptor protein *in vitro* and able to show if the changes in receptor activity measured in the whole animal by LDL clearance are controlled at the protein and, ultimately, at the DNA level.

Binding of ^{125}I -LDL to liver membranes has been extensively used as an assay for the liver receptor carried out *in vitro* (Kovanen *et al.*, 1979; Hui *et al.*, 1981; Kita *et al.*, 1981; Harders-Spengel *et al.*, 1982). However, these assays frequently produce variable results and only a small fraction of the binding of ^{125}I -LDL to liver membranes is due to the LDL receptor. More recently, binding of LDL to the LDL receptor separated by electrophoresis and transferred to nitrocellulose has been successfully used for the detection of the LDL receptor in

several tissues (Daniel *et al.*, 1983; Dresel *et al.*, 1984) including liver (Kroon *et al.*, 1984; Wade *et al.*, 1986). A limitation of both these assays, however, is that they first require isolation of liver membranes, a step which is associated with a variable and significant loss of receptor (see below).

We report here the purification to homogeneity of the LDL receptor from rabbit liver and the quantification of the receptor protein in whole tissue homogenates. This has been made possible by the availability of pure receptor protein and a newly developed anti-receptor monoclonal antibody which binds with high affinity and specificity detergent-solubilized LDL receptor.

The ability to measure the level of receptor protein and mRNA (Ma *et al.*, 1986) in liver and extra-hepatic tissues may help in the understanding of mechanisms which regulate the expression of the LDL receptor at the tissue level and especially in liver.

MATERIALS AND METHODS

Animals

Male and female (2.0–2.5 kg) New Zealand White rabbits were used. Treatment with 17α -ethinyloestradiol (Sigma, E 4876) was carried out subcutaneously for 7 days at a dose of 5 mg/kg as described by Kovanen *et al.* (1979).

Liver fractionation

Rabbits were killed by intravenous injection of sodium pentobarbitone (150 mg/kg) and the livers were isolated and washed in ice-cold 0.05 M-Tris/maleate/0.15 M-

Abbreviations used; LDL, low density lipoprotein (1.019–1.063 g/ml); HDL, high density lipoprotein (1.063–1.21 g/ml); PMSF, phenyl-methylsulphonyl fluoride; BSA, bovine serum albumin; TBS, Tris-buffered saline (0.025 M-Tris/HCl/0.15 M-NaCl, pH 7.4).

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NaCl/0.002 M-CaCl₂/0.001 M-PMSF, pH 6.5 (homogenization buffer). All subsequent operations were performed at 4 °C. Liver tissue was homogenized with a Polytron homogenizer (maximum setting, 15+15 s) at a concentration of 200 g of wet tissue per litre. The homogenate was centrifuged at 1000 g for 10 min and the supernatant centrifuged at 14000 g for 10 min. The 14000 g supernatant was centrifuged at 104000 g for 60 min in a Beckman 55 Ti rotor. The <1000 g, 1000–14000 g, and 14000–104000 g fractions were resuspended in homogenization buffer with a Potter homogenizer (3–4 strokes) and frozen in liquid N₂. In several experiments 14000–104000 g membranes were resuspended in 0.01 M-Tris/maleate/0.002 M-CaCl₂/0.001 M-PMSF, pH 6.5, and adjusted to 2.1 M-sucrose with 3.19 M-sucrose in 0.01 M Tris/maleate/0.002 M-CaCl₂/0.001 M-PMSF, pH 6.5, at a protein concentration of approx. 5 mg/ml. Membrane (11 ml) suspension in 2.1 M-sucrose was overlaid with 16 ml of 1.15 M-sucrose and 11 ml of 0.25 M-sucrose. Tubes were centrifuged for 4 h at 75000 g in Beckman SW28 rotor at 4 °C. Two major membrane subfractions were separated at the 2.1/1.15 M-sucrose interface (heavy subfraction) and at the 1.15/0.25 M-sucrose interface (light subfraction). These were collected, diluted to 0.25 M-sucrose with 0.01 M-Tris/maleate/0.002 M-CaCl₂/0.001 M-PMSF, pH 6.5, recovered by ultracentrifugation at 104000 g for 60 min in a 55 Ti rotor and processed as for the other membrane fractions.

Purification of the LDL receptor

Purification of the LDL receptor was carried out from the 14000–104000 g membranes using a combination of DEAE-Sephacel chromatography, LDL–Sephacel 4B chromatography and preparative SDS/polyacrylamide-gel electrophoresis. Membrane proteins were solubilized as described by Schneider *et al.* (1982) and the detergent extract in 0.05 M-Tris/maleate/0.05 M-NaCl/0.002 M-CaCl₂/0.001 M-PMSF/Triton X-100 (10 g/l), pH 6.0, was loaded on a column packed with DEAE-Sephacel (Pharmacia, 17-0500-01) and equilibrated in 0.05 M-Tris/maleate/0.05 M-NaCl/0.002 M-CaCl₂/0.001 M-PMSF/Triton X-100 (10 g/l), pH 6.0 (DEAE buffer A). Bound proteins were eluted with a gradient of NaCl (0.05–0.35 M) in 0.05 M-Tris/maleate/0.002 M-CaCl₂/0.001 M-PMSF/0.03 M-n-octyl- β -D-glucopyranoside, pH 6.0 (DEAE buffer B). Fractions containing the LDL receptor, as shown by LDL blotting, were pooled and designated DEAE-Sephacel fraction. This was dialysed for 36 h against 0.05 M-Tris/maleate/0.002 M-CaCl₂, pH 6.0, adjusted to pH 8.0 and loaded on an LDL–Sephacel 4B column equilibrated in 0.05 M-Tris/HCl/0.002 M-CaCl₂, pH 8.0. Bound proteins (LDL–Sephacel 4B fraction) were eluted with 0.05 M-Tris/maleate/0.0035 M-suramin (Bayer, lot FX214K), pH 6.0, and frozen in liquid N₂.

Final purification of the LDL receptor was carried out by preparative electrophoresis in SDS/polyacrylamide gels according to Walker *et al.* (1982). Slab gels (1.5 mm $\times$ 160 mm) were loaded with 52–209 μ g of protein (LDL–Sephacel 4B fraction). The LDL receptor band was cut and the receptor electro-eluted and collected in a dialysis tubing (cut-off point 10–14 kDa, Visking) as described by Walker *et al.* (1982). Purified receptor was dialysed for 24 h against 0.05 M-Tris/HCl/0.05 M-NaCl, pH 8.0 and stored at –70 °C.

Assays of LDL receptor

Binding of ¹²⁵I-LDL to liver membranes was carried out basically as described by Kovanen *et al.* (1979). Membrane protein (100 μ g) was used for each assay in 0.05 M-Tris/HCl/0.1 M-NaCl/BSA (20 g/l), pH 7.5, and incubated with ¹²⁵I-LDL for 60 min in ice at a concentration of 5 μ g of ¹²⁵I-LDL/ml. Membrane-bound ¹²⁵I-LDL was separated from free ¹²⁵I-LDL by a 5 min centrifugation in a Beckman Airfuge ultracentrifuge on a cushion of foetal bovine serum as described in Kovanen *et al.* (1979). For each membrane sample three tubes were incubated in the presence of 0.001 M-CaCl₂ (total binding) and three tubes were incubated in 0.005 M-EDTA (Ca²⁺-independent binding). The difference between the total binding and the Ca²⁺-independent binding of ¹²⁵I-LDL was regarded as the binding of ¹²⁵I-LDL to the LDL receptor.

Antibody and lipoprotein blots of LDL receptor were carried out after SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970) and transfer to nitrocellulose (Schleicher and Schuell, BA 85). Transfer was for 1 h at 0.5 A and 8–10 °C. Nitrocellulose was first blocked with 25 g of BSA/litre in TBS (blocking buffer) at 37 °C for 30 min. All subsequent incubations were carried out at room temperature and were in 25 g of BSA/litre in TBS plus 0.001 M-CaCl₂ unless otherwise indicated. For antibody blots nitrocellulose was incubated with anti-receptor monoclonal antibodies in blocking buffer followed by incubation with enzyme-conjugated second antibody and enzyme substrate. For LDL blots the paper was incubated with affinity-purified anti-LDL polyclonal or monoclonal IgG followed by incubation with enzyme-conjugated second antibody and enzyme substrate as indicated in the legends to Figs. 2 and 3. Between each incubation the paper was washed three times for 10 min with 1 g of Tween 20/litre in TBS (antibody blots) or with 2.5 g of BSA/litre in TBS (LDL blots).

A detergent-based double antibody assay for the LDL receptor was employed in some experiments. Flexible vinyl microtitre plates were coated overnight at room temperature with 5 μ g of anti-receptor monoclonal antibody/well in 0.05 ml of 0.05 M-Na₂CO₃/0.001 M-Na₃, pH 9.6. Plates were washed with TBS, blocked with 50 μ l of BSA (25 g/litre)/well in 0.05 M-Na₂CO₃ for 1 h at 37 °C and incubated for 3 h at room temperature with either purified LDL receptor or with different amounts of detergent extract of liver homogenate in 0.05 M-Tris/HCl/0.05 M-NaCl/0.002 M-CaCl₂/0.001 M-PMSF, 10 g/l Triton X-100, pH 6.8. Plates were washed three times with 1 g of Tween 20/litre in TBS, 250000 c.p.m. ¹²⁵I-MAC188 (see below) were added to each well and incubation was continued for 3 h. Plates were washed as above, air-dried and individual wells cut and counted.

Anti-LDL receptor monoclonal antibodies

Three rat monoclonal antibodies raised against purified rabbit liver LDL receptor were used in this study. They have been designated MAC188, MAC189 and MAC190. Monoclonal antibody MAC188 is an IgG₁; MAC189 and MAC190 are IgM. The antibodies were obtained by fusion of the spleen cells of a DA rat immunized with purified LDL receptor with the rat myeloma line Y3 Ag1.2.3 (Galfré *et al.*, 1979). A detailed characterization of these antibodies is to be reported separately.

Serum lipoproteins

LDL (1.019–1.063 g/ml) and HDL (1.063–1.21 g/ml) were isolated from rabbit and human serum by preparative ultracentrifugation in a 55 Ti rotor at 50000 rev./min for 16 h (LDL) or 24 h (HDL) at 4 °C. Lipoproteins were purified by recentrifugation at the same density, dialysed at 4 °C against 0.01 M-Tris/HCl/0.15 M-NaCl/0.001 M-EDTA, pH 7.4, and stored at 4 °C after filtration on 0.2 µm filters. LDL was conjugated to CNBr-activated Sepharose 4B (Pharmacia 17-04030-01) according to the instructions of the manufacturer using 25 mg of LDL protein/g of gel. Coupling efficiency ranged from 78 % to 93 %. Reductive methylation of LDL was carried out according to Weisgraber *et al.* (1978). Iodination of LDL (444–872 c.p.m./ng) with carrier-free ¹²⁵I (Amersham, IMS.30) was performed according to a modification of the procedure of Marchalonis (1969).

Other assays

Protein was assayed with a modification of the Lowry procedure (Markwell *et al.*, 1978). Samples containing 10 g of Triton X-100/litre or 0.03 M-n-octyl-β-D-glucopyranoside were diluted 10–100 times before assay. Samples eluted from the LDL-Sepharose 4B in 0.05 M-Tris/maleate/0.0035 M-suramin, pH 6.0, were assayed for protein after precipitation with trichloroacetic acid (50 g/l) in the presence of sodium deoxycholate (Schneider *et al.*, 1982). Apolipoproteins B and A-I in rabbit serum were measured by electroimmunoassay using sheep anti-sera raised against LDL (T 587) and apolipoprotein A-I (T 627).

RESULTS

LDL receptor specific activity in liver is 5–10 times lower than in adrenal glands (Brown *et al.*, 1979; Huettinger *et al.*, 1984), the tissue from which LDL receptor has previously been purified (Schneider *et al.*, 1982). Since administration of 17α-ethinyloestradiol to rats (Kovanen *et al.*, 1979) and rabbits (Kroon *et al.*, 1984; Ma *et al.*, 1986) was known to increase the expression of LDL receptor in liver, the hormone was administered for 7 days to adult rabbits in order to increase receptor specific activity in this tissue. The treatment produced a 24 % reduction in liver weight and a 3.5-fold increase of the liver receptor as measured in the membrane assay (Table 1). These changes were associated

with a fall in plasma apo B (49 %) and a less-pronounced reduction in serum apolipoprotein A-I (15 %). The increase in liver receptor produced by the hormone was less dramatic than the one obtained in other studies (Kovanen *et al.*, 1979; Ma *et al.*, 1986) but represented, nevertheless, a significant increase in LDL receptor specific activity in liver membranes.

To optimize the purifications of the LDL receptor from the liver of rabbits treated with 17α-ethinyloestradiol the distribution of the receptor among several subcellular fractions was studied and results are shown in Table 2. The 14000–104000 g membrane fraction contained approximately half the amount of LDL receptor present in liver homogenate. Subfractionation of this membrane fraction by sucrose density-gradient ultracentrifugation revealed that the highest LDL receptor activity was associated with the light membranes (Table 2). This subfraction is known to be enriched in plasma sinusoidal membranes (Evans, 1978) suggesting that the LDL receptor is preferentially localized on the sinusoidal side of the plasma membrane of the hepatocytes. A similar conclusion has been reached by electron microscopic studies of LDL uptake in the liver of rats treated with oestradiol (Chao *et al.*, 1981; Handley *et al.*, 1981). Although the light subfraction of the 14000–104000 g membranes appeared to be the best source of LDL receptor in liver, this fraction was not chosen for the purification of the receptor because of the reduced amounts of membranes which could be processed at each time on the sucrose gradient and the low recovery of receptor (Table 2). The 14000–104000 g fraction was finally used for the purification of the receptor protein.

Partial purification of the LDL receptor from liver was obtained by the DEAE and LDL-Sepharose 4B procedure of Schneider *et al.* (1982). Fig. 1 shows the elution profile of rabbit microsomal proteins eluted from a DEAE-Sephacel column with a gradient of NaCl in the presence of micellar concentrations of n-octyl-β-D-glucopyranoside. The receptor eluted from the column with a peak at 0.15 M-NaCl. The DEAE-Sephacel fraction (Fig. 1, bar) was subsequently applied on an LDL-Sepharose 4B column and the receptor eluted with a buffer containing 0.0035 M-suramin. While this procedure allowed purification to homogeneity of the LDL receptor from adrenal membranes (Schneider *et al.*, 1982), it yielded preparations of liver receptor containing variable amounts of other proteins. Under optimal conditions, the fraction eluted from LDL-Sepharose 4B contained the LDL receptor and two

Table 1. Effect of administration of 17α-ethinyloestradiol on liver weight and LDL receptor and serum apolipoprotein B and apolipoprotein A-I

Male New Zealand White rabbits (2.0–2.5 kg) received daily subcutaneous injections of 17α-ethinyloestradiol (5 mg/kg in propylene glycol) for 7 days. Control animals were injected with the solvent. At day 8 blood samples were taken for measurement of serum apolipoprotein B and apolipoprotein A-I by electroimmunoassay before animals were killed and liver membranes prepared and used for LDL binding as described under 'Materials and methods'. Data are means ± S.D. of values from five animals in each group.

	Liver weight (g)	Liver LDL receptor (ng of ¹²⁵ I-LDL bound/mg of protein)	Serum apo B (mg/l)	Serum apo A-I (mg/l)
Controls	84.0 ± 3.7	31.6 ± 12.5	125 ± 29	427 ± 50
Oestradiol	63.8 ± 5.8	110.6 ± 28.4	64 ± 24	365 ± 86

Table 2. Distribution of the LDL receptor among subcellular fractions in rabbit liver

Liver homogenate was prepared from male rabbits treated with 17α -ethinyloestradiol and membrane fractions were prepared at 1000 g, 14000 g and 104000 g. The 14000–104000 g membranes were subfractionated by sucrose-density ultracentrifugation into a heavy subfraction (2.1/1.15 M-sucrose) and a light subfraction (1.15/0.25 M-sucrose). Protein and LDL receptor were measured on each fraction. LDL receptor was measured by a modification of the LDL binding assay developed by Kovanen *et al.* (1979) (see the Materials and methods section for details). Data are means $\pm$ s.d. from the membrane preparations from three animals.

	Specific activity of LDL receptor (ng of 125 I-LDL bound/mg of protein)	Protein (g/fraction)	Total LDL receptor (μ g of 125 I-LDL bound/fraction)
Homogenate	27 ± 6	10.38 ± 0.75	280 ± 78
< 1000 g fraction	11 ± 6	1.92 ± 0.25	21 ± 13
1000–14000 g fraction	20 ± 7	2.24 ± 0.29	43 ± 9
14000–104000 g fraction	124 ± 48	1.19 ± 0.13	144 ± 44
Heavy subfraction	92 ± 29	0.68 ± 0.07	61 ± 14
Light subfraction	264 ± 81	0.11 ± 0.02	28 ± 8

Fig. 1. DEAE-Sephacel chromatography of 14000–104000 g soluble membrane proteins

Membrane proteins (14000–104000 g; 1.4 g) in 630 ml of 0.05 M-Tris/maleate/0.05 M-NaCl/0.002 M- CaCl_2 /0.001 M-PMSF, 10 g Triton X-100/litre, pH 6.0 (DEAE buffer A), were loaded on a 2.5 cm $\times$ 7.5 cm DEAE-Sephacel column at 60 ml/h. Unbound proteins (1.2 g) were collected as a single fraction and the column was washed with 75 ml of DEAE buffer A followed by 75 ml of 0.05 M-Tris/maleate/0.05 M-NaCl/0.002 M- CaCl_2 /0.001 M-PMSF/0.03 M-n-octyl- β -D-glucopyranoside (DEAE buffer B). Bound proteins were then eluted with a 150 ml linear gradient of NaCl (0.05–0.35 M) in DEAE buffer B at 30 ml/h. Fractions (3 ml) were collected and portions, assayed for protein and LDL receptor by LDL blots. Fractions containing LDL receptor (indicated with the bar in the figure) were pooled and subsequently used for LDL-Sephacel chromatography.

additional proteins with M_r values of 78×10^3 and 55×10^3 (results not shown).

Final purification of the receptor was achieved by preparative SDS/polyacrylamide-gel electrophoresis of the LDL-Sephacel 4B fraction followed by electroelution. Fig. 2 shows, on the left, Coomassie-stained SDS gels of the Triton X-100 extract of the 14000–104000 membranes (lane 1), the DEAE-Sephacel, un-

bound fraction (lane 2), the DEAE-Sephacel fraction (lane 3) and LDL receptor finally purified by LDL-Sephacel 4B followed by preparative gel electrophoresis (lane 4). The final yield of receptor was 20–25 ng/mg of liver protein. The M_r of the receptor protein was 120×10^3 – 125×10^3 in non-reducing gels (Fig. 2) and increased to 135×10^3 – 140×10^3 in reducing gels (results not shown). LDL blots of the same fractions are shown in Fig. 2 on the right. It is apparent that both the starting membrane extract and the DEAE-Sephacel fraction (lanes 1 and 3), in addition to the LDL receptor, contain a second LDL-binding protein to M_r of 270×10^3 . This protein has been observed by others (Hoeg *et al.*, 1986; Wade *et al.*, 1986) in LDL blots of liver membranes and Hoeg *et al.* (1986) have suggested that this protein is a liver-specific form of the LDL receptor. However, in our hands, the binding of LDL to this protein was not Ca^{2+} -dependent and the protein was not recognized by anti-receptor monoclonal antibody. Therefore, no further attempts were made to establish the identity of this protein.

Treatment of purified LDL receptor with neuraminidase changed both the M_r and the pI of the LDL receptor. The M_r shifted from 125×10^3 to 110×10^3 whereas the pI was raised from 4.45 to 5.25 (results not shown). Thus the rabbit LDL receptor, like the bovine one (Schneider *et al.*, 1982), contained sialic acid which accounted, in part, for its low pI .

Fig. 3 shows the ability of the purified liver LDL receptor to bind rabbit (lane 1) and human (lane 4) LDL. As expected, binding of rabbit LDL to the receptor was abolished in the absence of CaCl_2 (lane 2) and following reductive methylation of LDL (lane 3).

Using purified LDL receptor as antigen several rat anti-receptor monoclonal antibodies were produced. Three of these (designated MAC188, MAC189 and MAC190) were tested for their ability to quantify the LDL receptor in a double antibody solid-phase immunoassay. When purified LDL receptor was bound to immobilized monoclonal antibodies MAC188, MAC189 and MAC190 and probed with 125 I-MAC188, the amount of 125 I-MAC188 bound varied according to the combination of monoclonal antibodies used and the amount of LDL receptor per well. When MAC188 was

Fig. 2. SDS/polyacrylamide-gel electrophoresis and LD lipo-protein blots of rabbit liver LDL receptor at different stages of purification

Left, Coomassie-stained SDS gel of portions of soluble 14000–104000 *g* membranes (lane 1, 111 μ g of protein), DEAE-Sephacel unbound fraction (lane 2, 95 μ g of protein), DEAE-Sephacel fraction (lane 3, 89 μ g of protein) and purified LDL receptor (lane 4, 3.6 μ g of protein). Gel was run under non-reducing conditions. The mobility of M_r markers is indicated. Right, LDL blots of the same samples. Proteins were transferred to nitrocellulose and the paper was blocked. Blots were then incubated with rabbit LDL (5 μ g/ml), anti-rabbit LDL monoclonal IgG (MAC 23 at 5 μ g/ml) and alkaline phosphatase-conjugated second antibody (Sigma A9654, diluted 1/1000). Alkaline phosphatase was detected with the substrate described by Blake *et al.* (1984).

used both to immobilize and probe the receptor the amount of 125 I-MAC188 bound was small and up to 100 ng of LDL receptor/well were necessary to increase the counts at approximately twice the background. This indicated that the epitope recognized by MAC188 was not a multiple epitope. When MAC189 was used to immobilize the receptor and MAC188 used to probe the receptor, a linear relation between the amount of LDL receptor per well and the amount of 125 I-MAC188 bound was found in the range of 5–500 ng/well (results not shown). Thus, the double antibody solid-phase immunoassay using MAC189 and MAC188 was used to try to quantify the LDL receptor in detergent extracts of liver homogenate but failed to produce positive results. Three problems emerged with the tissue extracts: (1) the amount of 125 I-MAC188 bound was very low (< 200 c.p.m. of 125 I-MAC188 bound/well using the detergent extract from up to 250 μ g of protein); (2) it did not increase linearly with the amount of tissue extract used; and (3) when known amounts of LDL receptor were added to the detergent extract the amount of 125 I-MAC188 bound was less than that produced by the same amounts of LDL receptor in the absence of tissue extract.

Quantification of LDL receptor in liver was obtained using a quantitative blot assay based on MAC188 monoclonal antibody. Detergent extracts of liver homogenate were applied directly to SDS gels, transferred to nitrocellulose paper and probed with MAC188. In each experiment, known amounts of purified receptor were included to compensate for variations in transfer efficiency and detection of anti-receptor antibody. Under

Fig. 3. LDL blots of purified LDL receptor with rabbit and human LDL

Purified LDL receptor (0.6 μ g) was run on SDS gel (75 g/l) in a 3 cm slot, transferred to nitrocellulose and 0.5 cm strips (each containing 100 ng of LDL receptor) were cut, blocked and incubated with rabbit LDL (lanes 1 and 2), reductively methylated rabbit LDL (lane 3) and human LDL (lane 4) either in the absence or in the presence of 0.001 M-CaCl₂ as indicated in the Figure. Rabbit LDL was detected with rat anti-rabbit LDL monoclonal IgG as detailed in the legend to Fig. 2. Human LDL was detected with rabbit anti-human LDL polyclonal IgG followed by alkaline phosphatase-conjugated second antibodies and alkaline phosphatase substrate.

the conditions used the amount of MAC188 bound to the receptor depended solely upon the amount of LDL receptor present on the blot (Fig. 4, left) and the amount of receptor present in the whole homogenate could be accurately quantified by densitometry. The liver homogenate from four control female rabbits contained 149 ± 13 ng of LDL receptor/mg of liver protein. The liver homogenate of four female oestradiol-treated rabbits contained 312 ± 25 ng of LDL receptor/mg of protein.

DISCUSSION

In non-reducing gels purified LDL receptor from rabbit liver was found to be a glycoprotein of M_r

Fig. 4. Monoclonal antibody blots of LDL receptor in liver homogenate

Liver homogenates were prepared from eight female rabbits (2.0–2.5 kg body weight). Four rabbits were treated with 17 α -ethinyloestradiol for 7 days at 5 mg/kg. Homogenates were prepared as described in the Materials and methods section except that 0.05 M-Tris/HCl/0.15 M-NaCl/0.002 M-CaCl₂/0.001 M-PMSF, pH 6.8, was used as homogenization buffer. Liver proteins were solubilized with Triton X-100 (10 g/l final concn.) and the detergent extracts from 500 μ g of liver proteins were loaded on SDS gel (75 g/l), transferred to nitrocellulose and incubated with monoclonal antibody MAC188 (5 μ g/ml) followed by alkaline phosphatase-conjugated anti-(rat IgG) serum and alkaline phosphatase substrate. The first four lanes on the left show the blots of 32.25, 62.5, 125 and 250 ng of purified LDL receptor. These were quantified by densitometry and used to derive a calibration curve ($r = 0.997$) from which the amount of LDL receptor present in the tissue was calculated after densitometry.

120×10^3 – 125×10^3 (Figs. 2, 3 and 4), a figure close to previous estimates (128×10^3) based on ligand blotting experiments of crude liver membranes (Kroon *et al.*, 1984; Wade *et al.*, 1986). In similar ligand blotting experiments the M_r of the bovine adrenal receptor was found to be 130×10^3 (Daniel *et al.*, 1983), the M_r of the rat liver receptor was either 145×10^3 (Kroon *et al.*, 1984; Wade *et al.*, 1986) or 135×10^3 (Cooper *et al.*, 1987) and that of the dog liver receptor was 147×10^3 (Wade *et al.*, 1986). It is not presently clear to what extent these differences in M_r of the LDL receptor are due to differences in the amino acid sequence or in the carbohydrate moiety or both.

Considerable efforts have been made, in the last several years, to quantify the amount of LDL receptor in liver membranes by either binding experiments of 125 I-LDL or LDL blots of the LDL receptor. In our hands, however, these assays showed serious limitations when used with liver homogenates rather than with liver membranes. Indeed, although Kovanen *et al.* (1979) were able to quantify the binding of LDL to the LDL receptor in whole liver homogenate, we were not able to measure any Ca²⁺-dependent binding of 125 I-LDL in liver homogenates from normal rabbits and we could only detect low levels of receptor binding in animals treated with 17 α -ethinyloestradiol (Table 2). Similarly, when we tried the LDL blot assay on liver homogenate the level of non-receptor binding was unacceptable (results not shown). However, both assays can be successfully used with liver membranes.

We have found that preparation of the membranes

produces two results. First, LDL receptor specific activity is several fold higher in membranes compared with the whole homogenate (Table 2); secondly, the centrifugations at 1000 and 8000 g remove the greater proportion of the tissue components responsible for the non-receptor binding of LDL both in the tube or blot assays. However, in our experience, the preparation of liver membranes was associated with the loss of approximately half the LDL receptor of liver homogenate. This was a strong indication for the need for a receptor assay in the whole homogenate.

Unfortunately, the double antibody assay which performed well with purified receptor protein gave disappointing results when a detergent extract of liver homogenate was used. It is of interest that Knight *et al.* (1986) have recently succeeded in developing a double antibody immunoassay for the LDL receptor using partially purified receptor preparations instead of crude cell or tissue extracts. Using that procedure they found that human fibroblasts pre-incubated in lipoprotein-deficient serum contained 60 ng of LDL receptor/mg of protein (Knight *et al.*, 1986) and that human liver biopsies contained 20.2 ± 1.6 ng of LDL receptor/mg of liver membrane protein (Soutar *et al.*, 1986). This figure is lower than the one obtained in the present study (149 ± 13 ng/mg of liver protein) suggesting that the receptor content of rabbit liver is much higher than that of human liver.

The major disadvantage of the receptor assay reported in this study, compared with the double antibody assay in microtitre plates, is the limited number of samples which can be processed at one time. Typically, we load purified receptor on to four lanes and up to ten samples onto each gel. One advantage of the blot assay, however, is that the LDL lipoprotein receptor precursor (the faint band with an M_r of 90×10^3 in Fig. 4) can be quantified, if required, separately from the mature form of the receptor. This can be easily achieved (at least with liver or spleen tissues) by loading the detergent extract from 1 mg of protein (results not shown).

The assay for the LDL receptor in liver homogenate which we have developed may prove useful in the study of the factors which regulate the expression of the LDL receptor in liver. Evidence is available from animal studies that dietary lipids suppress the expression of receptor in liver (Kovanen *et al.*, 1981; Mahley *et al.*, 1981; Spady & Dietsch, 1985) and that suppression of the liver receptor may be partially reversed by hormones (Kushwaha & Hazzard, 1981) and drugs (Chao *et al.*, 1983). Administration of 17 α -ethinyloestradiol to the rabbit increases the amount of mRNA for the receptor (Ma *et al.*, 1986), the binding of 125 I-LDL to liver membranes (Table 2) and the amount of receptor protein in the tissue (Fig. 4). With the ability to quantify steady-state levels of LDL receptor and LDL receptor mRNA it should now be possible to study the effect of major physiological factors, including age, diet and hormonal balance, on the expression of the LDL receptor in liver. Such studies may not only clarify the role of the liver LDL receptor in regulating serum levels of LDL but may well facilitate the search for new drugs for the treatment of hypercholesterolaemia.

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