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Immunoblotting and ligand blotting of the low-density lipoprotein receptor of human liver, HepG2 cells and HeLa cells

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Low-density lipoprotein receptors from adult human liver and the human hepatoblastoma cell line HepG2 were analyzed by polyacrylamide electrophoresis in SDS followed by immuno- and ligand botting. In both liver and HepG2 we detected a protein band with apparent relative molecular mass of 130 kDa, which is similar to that of the LDL receptor in fibroblasts. In addition we showed that HeLa cells also possess this LDL-receptor protein.

Apoliprotein B receptor; Apoliprotein E receptor; LDL; IDL; (Human liver, HepG2 cell, HeLa cell)

1. INTRODUCTION

The liver is the most important organ in cholesterol homeostasis, since it can irreversibly remove large quantities of cholesterol from the circulation [1,2]. In animal models it has been shown that the majority of the LDL-uptake in the liver is receptor-mediated [3]. Furthermore, the presence of LDL receptors on human liver membranes [4] and on plasma membranes of the human hepatoblastoma-derived cell line HepG2 [5,6] has been demonstrated. LDL receptors from human fibroblasts, A431 cells and rabbit and bovine adrenal tissue have been visualised by immuno- and ligand blotting techniques after polyacrylamide electrophoresis [7,8]. Wade et al. [9] recently used ligand blotting to detect LDL receptors in the liver from rabbit, rat and dog. It was further shown that these receptors have apparent relative molecular masses ranging from 128 (rabbit) to 147 kDa (dog) (non-reducing electrophoresis conditions). The quantity and molecular mass of LDL receptors in adult human liver has, however, been a matter of controversy [10,11]. Hoeg et al. [12] reported the presence of two distinct LDL-binding proteins in human liver with apparent M_r values of 270000 and 330000. They did not detect a receptor protein of apparent molecular mass corresponding to that of LDL receptors of fibroblasts or adrenal glands. In the present work we show that adult human liver does indeed contain LDL receptors with characteristics very similar to those of the fibroblast receptor. We also demonstrate the presence of this receptor protein in HepG2 and HeLa cells.

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Abbreviations: LDL, low-density lipoproteins; IDL, intermediate-density lipoproteins; VLDL, very low-density lipoproteins; apo B, apolipoprotein B; apo E, apolipoprotein E; apo E/PC, phospholipid complexes containing apo E

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2. MATERIALS AND METHODS

2.1. *Solubilization of cultured cells*

Fibroblasts, HeLa cells or HepG2 cells were grown on medium containing 10% lipoprotein deficient serum for two days before harvesting with a rubber scraper. The LDL receptor was extracted by solubilizing the cells with 40 mM octyl-β-D-glucoside in buffer A (50 mM Tris/maleate, pH 6.5, 2 mM $CaCl_2$, 1.5 mM PMSF, 0.1 mM leupeptin) for 10–15 min on ice. The extract was clarified by ultracentrifugation at $6 \times 10^6 \times g \cdot$min.

2.2. *Preparation and solubilization of human liver membrane extracts*

Human liver was obtained from hepatic tumor surgery, using only redundantly removed healthy tissue. For one experiment, a liver specimen was used from a patient homozygous for familial hypercholesterolemia, who did not express the fibroblast LDL receptor; this material, which came available after liver transplantation, was kindly provided by Dr H.B. Brewer (NIH, Bethesda). The tissue was put immediately into ice-cold isotonic saline, cut into small pieces and either frozen in liquid nitrogen or used immediately.

All subsequent operations were carried out at 0–4°C. The tissue was placed in buffer B (10 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$ and 1 mM PMSF; 5 ml/g tissue) and homogenized with two 15-s pulses of a Polytron homogenizer (setting 10). The homogenate was filtered through Nylon cloth (120 μm mesh) and centrifuged at $500 \times g$ for 5 min. The supernatant was centrifuged at $8000 \times g$ for 15 min, the resulting postmitochondrial supernatant then being ultracentrifuged for 60 min at $100000 \times g$. The pellet was resuspended in buffer B, using successively a 22-gauge and a 25-gauge needle, and centrifuged again at $100000 \times g$ for 60 min. Finally, the membrane pellet was stored in liquid nitrogen or immediately solubilized.

For solubilization, membrane pellets were resuspended in buffer A (1–2 ml/g of starting wet liver tissue). The suspension was sonicated with an MSE sonicator (two 5-s pulses, medium power, ampl. 3). An equal volume of 80 mM octyl-β-D-glucoside in buffer A was added and the sample was allowed to solubilize for 10–15 min on ice with occasional stirring. The extract was clarified by ultracentrifugation at $6 \times 10^6 \times g \cdot$min.

2.3. *Electrophoresis and blotting*

Samples of solubilized cells or liver membrane extracts were mixed with an equal volume of 2 × conc. sample buffer (without β-mercaptoethanol) for SDS electrophoresis, either according to the system of Laemmli [13] or the system of Neville [14]. Bio-Rad high molecular mass standards were reduced by heating at 95°C for 5 min in sample buffer containing β-mercaptoethanol. After gel electrophoresis, proteins were transferred electrophoretically to nitrocellulose (Schleicher & Schüll, 0.45 μm) for 16–18 h at 200 mA (blotting buffer: 20 mM Tris-base/150 mM glycine, 20% methanol). The blot was saturated in 5% bovine serum albumin in 50 mM Tris-Cl, 90 mM NaCl, 2 mM $CaCl_2$ at pH 8 (4–6 h at 4°C). For immunodetection, strips of the blot were incubated overnight at 4°C with polyclonal rabbit anti-bovine LDL-receptor antibodies or non-immune rabbit IgG (control). Incubation buffers and washing procedures were essentially as described [7]. Bound antibodies were detected with peroxidase-conjugated goat anti-rabbit IgG (Jackson) and 4-chloronaphthol/H_2O_2. For ligand binding, blot strips were incubated overnight with human LDL (40–80 μg/ml), human IDL (40–80 μg/ml) or apo E/phospholipid complexes (3 μg protein/ml). For controls, Ca^{2+} was replaced by 10 mM EDTA in the ligand incubation buffer. Bound ligand was detected either by incubation with rabbit antibodies against apo B or apo E, or hybridoma supernatant containing monoclonal antibodies against apo E (Beisiegel et al., in preparation), followed by incubation with goat anti-rabbit (or anti-mouse) IgG-peroxidase conjugate and substrate as described above. Incubation buffers and washing procedures were according to the method described by Daniel et al. [8].

2.4. *Preparation of lipoproteins*

LDL and IDL were prepared from fresh frozen plasma from normal or hyperlipidemic donors. After centrifugation of the lipoprotein fraction (d = 1.21 g/ml) in a modified Redgrave gradient [15] the LDL was taken from the density range d = 1.050–1.060 g/ml and IDL from the density range d = 1.019–1.035 g/ml.

2.5. *Formation of apo E/phospholipid complexes (apo E/PC)*

VLDL was prepared from hyperlipidemic plasma by ultracentrifugation at density d = 1.006 g/ml for 20 h at 100000 × g and respun under the same conditions. After delipidation with acetone/ethanol (1:1, v/v) the apo-VLDL was subjected to preparative electrophoresis in the Neville system [14]. The apo E band was electro-eluted from the polyacrylamide using the elution columns of a Desaga electrophoresis chamber. The product was dialyzed extensively against 10 mM Tris-Cl buffer, pH 7.4, containing 140 mM NaCl and 1 mM EDTA (buffer C) to remove dodecylsulphate. A sample of the purified apo E was used for the formation of apo E/PC according to the method described by Helenius et al. [16] as modified by Schneider et al. [17]. Briefly, a lipid film was formed in a glass tube by evaporating a solution of 2 mg phosphatidylcholine (Serva) and 40 μg cholesterol (Serva) in chloroform/ethanol under a nitrogen stream. 220 μg apo E, mixed with 23.4 mg of sodium cholate in buffer C, were added to the lipid film, which was then vigorously vortexed for 1 min and incubated at 24°C for 20 min prior to extensive dialysis against buffer C.

3. RESULTS

Octylglucoside extracts of HeLa cells were subjected to electrophoresis according to Laemmli [13]. The proteins were transferred onto nitrocellulose. A protein band of apparent M_r 130000 was detected which specifically bound anti-LDL-receptor antibodies. The same protein band interacted with LDL in a Ca^{2+}-dependent manner (fig.1). The relative electrophoretic mobilities of the LDL receptors from human skin fibroblasts or HeLa cells showed no significant difference.

We next used the immuno- and ligand blotting techniques on blot strips of octylglucoside-extracted human liver membranes. HeLa cell extracts were used for comparison. With polyclonal antibodies to bovine LDL receptor we detected a single band of apparent M_r 130000 on blot strips from both HeLa extracts and human liver membrane preparations. The LDL-receptor band of liver membranes was very strong in comparison to that of HeLa cells, also relative to the total amount

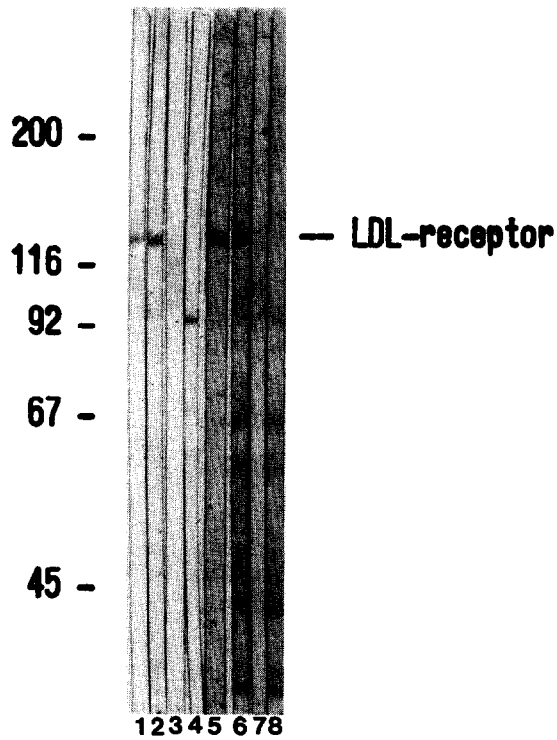

Fig.1. Immuno- and ligand blots of human skin fibroblasts and HeLa cells. Octylglucoside extracts of fibroblasts and HeLa cells were subjected to SDS-polyacrylamide electrophoresis and then electroblotted onto nitrocellulose. Lanes: 1, fibroblasts, anti-LDL-receptor immunoblot; 2, HeLa cells, anti-LDL-receptor immunoblot; 3, fibroblasts, non-immune rabbit IgG conotrol; 4, HeLa cells, non-immune rabbit IgG control; 5, fibroblasts, LDL ligand blot; 6, HeLa cells, LDL ligand blot; 7, fibroblasts, LDL ligand blot + EDTA; 8, HeLa cells, LDL ligand blot + EDTA. Molecular mass markers (kDa): myosin (200); β-galactosidase (116); phosphorylase b (92); bovine serum albumin (67); ovalbumin (45).

of extracted protein, applied to the gel (fig.2a). By applying the ligand blot procedure to these strips (same electrophoresis and blotting run), using either LDL or IDL as ligand, we detected the same 130 kDa protein (fig.2b). LDL produced a somewhat weaker band than IDL, while the difference in intensity of the LDL-receptor band between HeLa and liver membrane strips was similar to that observed in the immunoblot procedure. Incubation in the presence of 10 mM EDTA

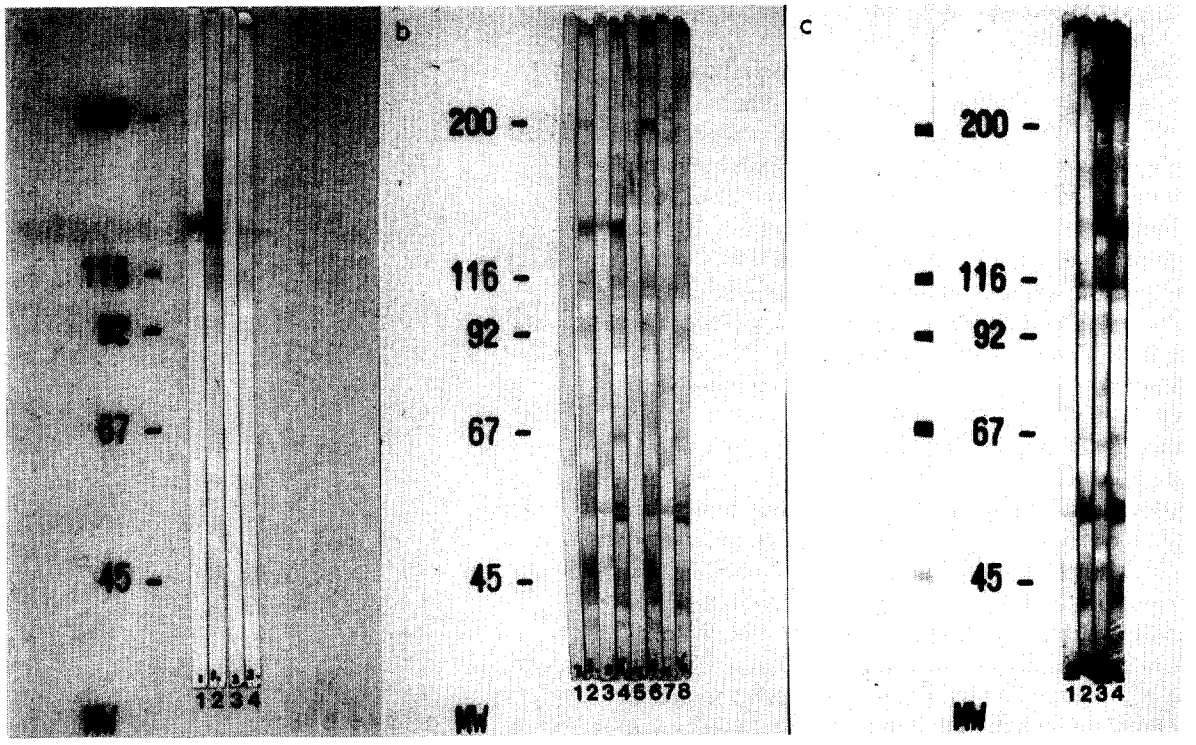

Fig.2. Immuno- and ligand blots of human liver membranes. Octylglucoside extracts of human liver membranes were subjected to electrophoresis and blotting. Blots represent approx. 85 μg extracted protein/blot strip (3 mm width). HeLa blot strips were used for comparison (25 μg extracted protein/strip). (a) Anti-LDL-receptor immunoblots. Lanes: 1 and 3, HeLa cells; 2 and 4, human liver membranes; 1 and 2, anti LDL-receptor; 3 and 4, non-immune rabbit IgG. (b) LDL and IDL ligand blots. Lanes: 1, 3, 5 and 7, HeLa cells; 2, 4, 6 and 8, human liver membranes; 1 and 2, LDL, anti-apo B; 3 and 4, IDL, anti-apo E; 5 and 6, LDL + EDTA, anti-apo B; 7 and 8, IDL + EDTA, anti-apo E. (c) Apo E/PC ligand blots. Lanes: 1 and 3, HeLa cells; 2 and 4, human liver membranes; 1 and 2, apo E 2/2 PC complexes, anti apo E; 3 and 4, apo E 3/3 PC complexes, anti apo E. Molecular mass markers are as in fig.1.

prevented the binding of LDL and IDL to the 130 kDa protein (fig.2b), showing that the interaction is Ca^{2+}-dependent, as expected for the LDL receptor. In the ligand blotting experiment illustrated in fig.2c we used phospholipid complexes containing apo E as ligand. Complexes containing the apo E isoform 3/3 bound to LDL receptors on blots. However, no LDL receptor band was detected with complexes containing apo E2/2, a genetic variant which is not recognized by the LDL receptor [17] (fig.2c). Controls were performed with phospholipid complexes (apo E3/3) in the presence of EDTA and with phospholipid complexes produced with bovine serum albumin instead of apo E (not shown).

We used the polyclonal rabbit anti-LDL-receptor antibody to test liver membranes from a patient who did not express the fibroblast LDL receptor (homozygous familial hypercholesterolemia). In these liver membranes we could not detect any LDL receptor with the immunoblot procedure. The same amount of normal liver membrane protein did show the 130 kDa LDL-receptor protein in control immunoblots (not shown).

Experiments similar to those described above were carried out with extracts of HepG2 cells, using the electrophoresis system of Neville [14]. Again, a protein band of apparent M_r 130000, having immunological and ligand binding properties characteristic of LDL receptors could be detected (fig.3). In these experiments the use of a monoclonal anti-apo E antibody as second step in

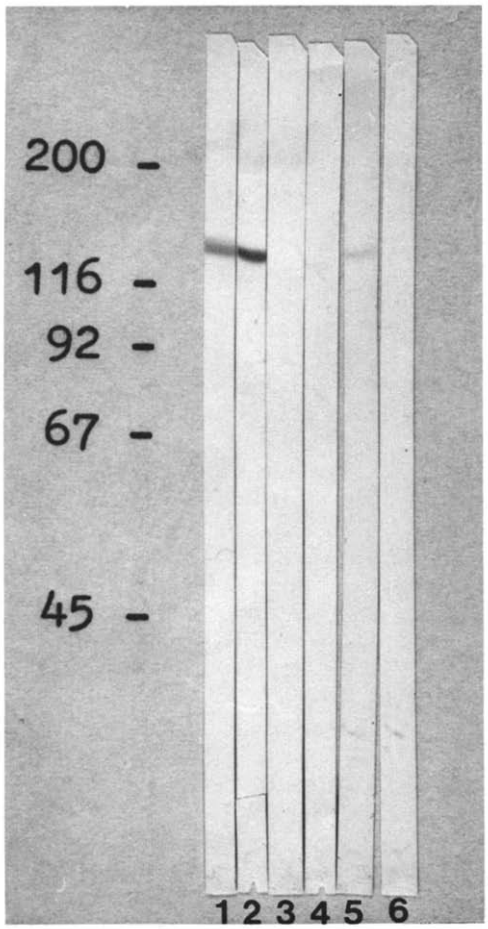

Fig.3. Immuno- and ligand blots of HepG2 cells. Lanes: 1, 3, 5 and 6, HepG2 cells; 2 and 4, HeLa cells; 1 and 2, anti-LDL-receptor; 3 and 4, non-immune rabbit IgG; 5, IDL, monoclonal anti-apo E (hybridoma supernatant); 6, control without monoclonal anti-apo E. Molecular mass markers are as in fig.1.

the ligand incubation procedure led to results essentially free from background staining (cf. figs 2 and 3).

4. DISCUSSION

We have analyzed the LDL receptor in extracts of HeLa cells, human hepatoblastoma HepG2 cells and human liver membranes. HeLa cells were shown to possess LDL receptors with an apparent M_r similar to that of normal human cultured fibroblasts, as detected on nitrocellulose blots (fig.1). Binding, uptake and degradation of LDL by HeLa cells display the characteristics of specific, receptor-mediated endocytosis (Beisiegel, U., unpublished). HeLa cells therefore provide a valid and convenient tissue culture model for studying ligand-receptor interactions of the LDL receptor. In the present investigation we used the LDL receptor of HeLa cells as reference material in blotting experiments.

Binding and uptake of LDL in the human liver is mediated, at least in part, by LDL receptors [4,11,18]. In this paper we have shown that these receptors can be detected as a protein band of apparent M_r 130000 on nitrocellulose blots after octylglucoside extraction, electrophoresis under non-reducing conditions and blotting. No 130 kDa LDL receptor could be detected by immunoblotting using liver membranes from a patient lacking the fibroblast LDL receptor (homozygous familial hypercholesterolemia).

Our findings, for the human liver, complement those of Wade et al. [9] on hepatic LDL-receptors of dog, rat and rabbit. Our results differ from those of Hoeg et al. [12], who detected ^{125}I-LDL-binding proteins of M_r 270000 and 330000 in human liver. As suggested by these authors, these bands may as well represent other proteins with LDL-binding activity. It remains unclear why they did not detect the 130 kDa LDL receptor. Variations in hepatic LDL-receptor content between different individuals may play a role in explaining this discrepancy. In our experiments, however, we obtained essentially identical results with liver membranes from two different subjects (male, 59 years old, hepatic tumor surgery, shown in fig.2; female, 34 years old, healthy donor, not shown). In neither case did the quantity of LDL receptors found in our liver membrane extracts provide an obstacle to detection. Moreover, in a personal communication Dr Brewer confirmed that in recent experiments the 130 kDa LDL receptor of human liver was indeed detected in his laboratory.

In accordance with the results obtained for human liver, we showed in this paper that human hepatoblastoma HepG2 cells also express an LDL-receptor protein of M_r 130000. Our ligand blotting and immunoblotting experiments with the LDL receptor in HepG2 cells confirm earlier results of other laboratories, showing that HepG2 cells bind, internalize and degrade LDL via a receptor-

mediated pathway with kinetics similar to that of LDL receptors from extrahepatic cells [5,6,18].

Although hepatocytes certainly contribute to a large extent to the LDL binding capacity of the liver, the relative contribution of hepatocytes, Kupffer cells and hepatic endothelial cells to the amount of LDL receptors contained in liver membrane extracts cannot be determined from the present experiments.

We conclude that LDL receptors in HeLa cells, HepG2 cells and human liver membranes, with respect to molecular mass and antibody- and ligand-binding specificities on nitrocellulose blots, are indistinguishable from the fibroblast LDL receptor. Thus, these proteins are probably all products of the same human gene.

During the preparation of this article an independent study reported the detection and quantitation of LDL receptors in human liver by ligand blotting, immunoblotting, and radioimmunoassay [20].

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