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The human asialoglycoprotein receptor is a possible binding site for low-density lipoproteins and chylomicron remnants

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Binding and internalization of chylomicron remnants from rat mesenteric lymph by HepG2 cells was inhibited by both excess remnants and low-density lipoprotein (LDL) to the same extent. Ligand blots revealed binding of remnants and LDL to the LDL receptor. Measures regulating LDL receptor activity greatly influenced the binding of remnants: ethinyloestradiol, the hydroxymethylglutaryl-CoA reductase inhibitor pravastatin and the absence of LDL all increased binding, whereas high cell density or the presence of LDL decreased binding. Also, asialofetuin, asialomucin, the neoglycoprotein galactosyl-albumin and an antibody against the asialoglycoprotein receptor all decreased substantially the binding of remnants. At high cell density, binding, internalization and degradation of chylomicron remnants was inhibited by up to 70–80%, yet binding of LDL was inhibited by no more than 20–30%. In cross-competition studies, the binding of ^{125}I-asialofetuin was efficiently competed for by asialofetuin itself or by the antibody, and also by LDL and remnants, yet remnants displayed an approx. 100-fold higher affinity than LDL. Likewise, remnants of human triacylglycerol-rich lipoproteins and asialofetuin interfered with each others' binding to HepG2 cells or human liver membranes. It is concluded that the LDL receptor mediates the internalization of chylomicron remnants into hepatocytes depending on its activity, according to demand for cholesterol. Additionally, the asialoglycoprotein receptor may contribute to the endocytosis of LDL, but predominantly of chylomicron remnants.

INTRODUCTION

Lipoproteins containing apolipoprotein B are largely taken up by hepatic parenchymal cells (Chao *et al.*, 1981; Jones *et al.*, 1984; Dietschy & Spady, 1986; Nenseter *et al.*, 1988). Two-thirds of low-density lipoprotein (LDL) has been calculated to be removed from the circulation by the LDL receptor, and the remainder via less-well-defined mechanisms (Goldstein & Brown, 1982; Pittman *et al.*, 1982). In accordance with these observations, LDL accumulates in states of lowered or absent LDL receptor activity (Goldstein & Brown, 1982).

However, mechanisms of hepatic removal of chylomicron remnants are a subject for debate, since there is no indication of an accumulation of chylomicron remnants in patients with homozygous familial hypercholesterolaemia. Also, in rabbits deficient in the LDL receptor, the removal of chylomicron remnants is indistinguishable from that in normal rabbits. The underlying genetic defect leads to a decreased number of LDL receptors without affinity for LDL; however, these may still bind and internalize apolipoprotein-E-containing lipoproteins (Kita *et al.*, 1982; Yamamoto *et al.*, 1986; Wernette-Hammond *et al.*, 1989). Antibodies to the LDL receptor resulted in only incomplete competition for the binding of chylomicron remnants to the surface of cultured hepatocytes, and down-regulation of the LDL receptor in experimental animals did not affect remnant clearance (Hui *et al.*, 1981; Cooper *et al.*, 1987). Further evidence for a separate receptor for chylomicron remnants is provided by the observation of the suppression of binding and uptake of chylomicron remnants, but not of LDL, by lipoprotein-X (Walli & Seidel, 1984). Thus the contribution of the LDL receptor to the removal of chylomicron remnants has been questioned, and the existence of alternative removal mechanisms has been postulated.

We have shown by competition experiments that LDL and chylomicron remnants share common binding sites on rat liver membranes, and ligand blots indicated binding of both particles to the LDL receptor (Windler *et al.*, 1988). This is in line with the finding of identical intracellular pathways for LDL and chylomicron remnants and the fact that antibodies directed against the LDL receptor inhibit the binding of chylomicron remnants to endosomal membranes (Jones *et al.*, 1984; Jaeckle *et al.*, 1989). A cell-surface protein of approx. 500 kDa has been identified which contains reiterating ligand-binding domains of the LDL receptor (Herz *et al.*, 1988). However, a physiological function of this protein in the endocytosis of lipoproteins, particularly that of chylomicron remnants, has not yet been established (Kowal *et al.*, 1989; Lund *et al.*, 1989).

We have considered lectins as candidates to mediate the unimpaired removal of chylomicron remnants in states of LDL receptor deficiency. Lectins are involved in numerous biological interactions of proteins and cells (Ashwell & Harford, 1982). The apolipoproteins B and E have been shown to be highly glycosylated (Jain & Quarfordt, 1979; Taniguchi *et al.*, 1989). Human apolipoprotein B was reported to contain, besides high-mannose-type oligosaccharides, about 60% biantennary complex and hybrid-type oligosaccharides, which may function in the asialo form as ligands for galactose-recognizing lectins (Filipovic *et al.*, 1979; Orekhov *et al.*, 1989). Post-translational *O*-glycosylation has been described for rat and human apolipoprotein E (Reardon *et al.*, 1984; Zanni *et al.*, 1989). The di-, tetra- or hexa-sialylated form is subsequently desialylated in plasma, and a physiological role for the galactosyl residues has been suggested (Zannis *et al.*, 1984; Hussain *et al.*, 1988). We have carried out analyses of the content of galactosyl and sialo residues in apolipoproteins of chylomicron remnants and LDL and the effect of the modification of these terminal carbohydrates

Abbreviations used: ASGP, asialoglycoprotein; LDL, low-density lipoprotein; HMG-CoA, hydroxymethylglutaryl-CoA.

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on binding and uptake. Preliminary results indicate that apolipoproteins B-100, B-48 and E of human LDL and rat chylomicron remnants as prepared in this investigation contain terminal galactose (E. Windler & A. Block, unpublished work). Thus the asialoglycoprotein (ASGP) receptor, a binding site abundant in hepatocytes (Schwartz *et al.*, 1982; Geuze *et al.*, 1986), might be involved in the removal of lipoproteins. This study has been designed to investigate whether this receptor can contribute to the hepatic uptake of chylomicron remnants as well as the LDL receptor, the principal binding site for lipoproteins.

MATERIALS AND METHODS

Animals and materials

Rat plasma was obtained from male Sprague–Dawley rats (Zentrale Versuchstieranstalt, Hanover, Germany), weighing 250–350 g and fed on normal laboratory chow and tap water (Windler *et al.*, 1988). BSA (fraction V), biotin hydrazide, sodium cyanoborohydride, Naphthol Blue-Black, asialofetuin, asialomucin and transferrin were obtained from Sigma, St Louis, MO, U.S.A. Human serum albumin was from Behringwerke, Marburg, Germany, and pravastatin was from Squibb & Sons, Munich, Germany. Galactosyl–BSA (Biomol, Hamburg, Germany) contained 30–40 mol of 2-(2-carbomethoxyethylthio)-ethyl β-D-galactopyranoside/mol of BSA. Sodium metaperiodate, H_2O_2, 4-chloro-1-naphthol and Triton X-100 were purchased from E. Merck, Darmstadt, Germany. Phosphate-buffered saline (sterile, pH 7.4), Dulbecco's modified Eagle's medium, fetal calf serum, trypsin-EDTA, penicillin/streptomycin, neomycin, Hepes buffer and L-glutamine were from Gibco, Paisley, Scotland, U.K. Tris buffer contained 50 mM-NaCl, 1 mM-$CaCl_2$ and 20 mM-Tris, pH 7.4. Preparation of a monoclonal antibody directed against the rat ASGP receptor (28 D6), which cross-reacted with the human ASGP receptor, is described in detail elsewhere (Roos *et al.*, 1985; Treichel *et al.*, 1989). Tissue culture flasks (175 cm^2) were purchased from Nunc, Wiesbaden, Germany, and tissue culture wells (2.2 cm diam.) were from Costar, Cambridge, U.K. Radioactivity was counted in a γ-radiation counter (Packard, Frankfurt, Germany), or in 10 ml of scintillation fluid (Rotizint; Roth, Karlsruhe, Germany) in a β-radiation counter (Packard), with a counting error of less than 5%.

Preparation of lipoproteins

Preparation and characterization of lipoproteins has been described in detail (Windler *et al.*, 1988). For preparative ultracentrifugation, Ti 60, 50, and 50.3 rotors (Beckman, Munich, Germany) were used. Human LDLs were prepared by sequential ultracentrifugation in the density range $d = 1.024$–1.050, and small chylomicrons, in some experiments labelled with [1,2-^{3}H]-cholesterol (Amersham–Buchler, Braunschweig, Germany), were isolated from rat mesenteric lymph as described (Windler *et al.*, 1988). To produce rat chylomicron remnants as characterized in Windler *et al.* (1988), small chylomicrons were incubated in post-heparin plasma free of very-low-density lipoprotein at 37 °C for 15 min as described (Windler *et al.*, 1988). To produce remnants of human triacylglycerol-rich lipoproteins, serum was drawn from a normolipaemic subject 4 h after a meal of 500 ml of cream (30% fat) and centrifuged for 8×10^7 $g_{av.} \cdot$min after raising the density to $d = 1.019$ by the addition of H_2O according to Windler *et al.* (1986). The top fraction was incubated with human post-heparin plasma, produced as described by Windler *et al.* (1986), at a final concentration of 1 mg of triacylglycerol/ml for 15 min at 37 °C. By centrifuging twice under the above conditions, human remnants were isolated in the top fraction. For labelling with ^{125}I ($Na^{125}I$ 480–630 MBq/μg of iodide, carrier-free, Amersham–Buchler), the iodine monochloride method of McFarlane adapted for lipoproteins was applied as described (Windler *et al.*, 1988), with the addition of two passages through columns of Sephadex G-25 M (PD-10 columns; Pharmacia, Uppsala, Sweden). More than 97% of radioactivity was precipitable in 10% trichloroacetic acid, and of the radioactivity in chylomicron remnants, 72.5 ± 7.4% ($n = 4$) was extractable into organic solvents (Bligh & Dyer, 1959). The specific radioactivity ranged from 80000 to 200000 c.p.m./μg of protein. The ratio of cholesteryl esters/protein (w/w) was 2.0 ± 0.5 for LDL ($n = 6$) and 0.5 ± 0.1 for chylomicron remnants ($n = 6$).

Membrane binding assay

Preparation of liver membranes and membrane binding assays were performed exactly as described, using Ti 60 and LP 42 rotors (Windler *et al.*, 1988).

Cell culture

HepG2 cells were grown in culture flasks in Dulbecco's modified Eagle medium to which final concentrations of 10% (v/v) fetal calf serum, 100 units of penicillin/ml, 100 μg of streptomycin/ml, 100 μg of neomycin/ml and 2 mM-L-glutamine had been added. Cells were washed in phosphate-buffered saline and trypsin-treated at 24 °C for 10 min. The reaction was stopped by the addition of 2 vol. of medium. Cells were immediately pelleted by 10^3 $g_{av.} \cdot$min at 4 °C. If not stated otherwise, 10^6 cells in 1 ml of medium were seeded on to tissue culture wells and grown for 48 h to confluence. In experiments with labelled lipoproteins as ligands, the above culture medium was changed to one without fetal calf serum 24 h before experiments.

Cell binding assay

Cells were incubated in 250 μl of medium containing various ligands at 37 °C or 4 °C for 1 h. The supernatant was removed and the cells were rinsed with 4×500 μl of phosphate-buffered saline. For determination of cell-associated ligands, cells were lysed in 1 M-NaOH for 10 min at 24 °C and transferred to counting vials. The radioactivity, corrected for background, was taken as a measure of lipoproteins associated with cells, which was expressed as the component cholesteryl esters. To allow results to be expressed as a function of cell protein, the protein in each dish was measured when ^{125}I was used or, in experiments with tritiated lipoproteins, the protein in control dishes was determined. To discriminate bound and internalized ligand, cells were treated with trypsin at 37 °C after washing (Dashti *et al.*, 1984). After about 5 min, 1 vol. of ice-cold phosphate-buffered saline was added and the cell suspension plus one wash of the dish with 500 μl of ice-cold phosphate-buffered saline were centrifuged at 2.5×10^4 $g_{av.} \cdot$min at 4 °C. The radioactivity in the supernatant and in the pellet was taken as a measure of the bound and internalized ligand respectively. Control experiments using a dextran-sulphate-containing buffer [50 mM-NaCl, 4 mg of dextran sulphate 500 (sodium salt)/ml (Serva, Heidelberg, Germany) and 10 mM-Hepes, pH 7.4] to remove ligand from its receptor as described (Goldstein *et al.*, 1976) yielded comparable results. Degradation was measured in pulse–chase experiments. HepG2 cells were incubated with iodinated ligands at 4 °C for 1 h, rinsed as described above and incubated with 500 μl of medium at 37 °C for indicated times. Non-protein-bound radioactivity was measured in the supernatant and in one wash of the cells with phosphate-buffered saline after precipitation of the protein by trichloroacetic acid. Requirement of Ca^{2+} for binding was determined according to Schwartz *et al.* (1981), with the addition of six rinses with phosphate-buffered saline containing

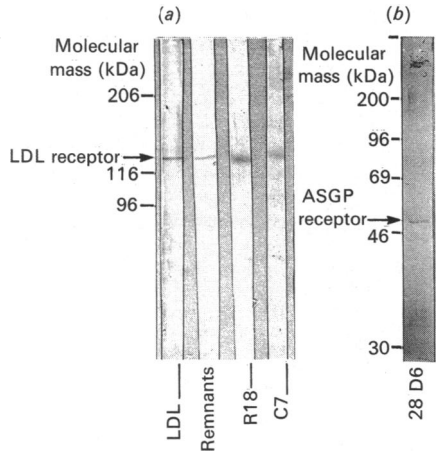

Fig. 1. Ligand and immunoblots of the LDL receptor and an immunoblot of the ASGP receptor of HepG2 cells

Proteins of HepG2 cells were subjected to SDS/PAGE and transferred to nitrocellulose membranes. Receptor-proteins were detected by biotinylated lipoproteins or by polyclonal (R48) or monoclonal (C7) antibodies directed against the LDL receptor (*a*) or a monoclonal antibody (28 D6) against the ASGP (*b*).

10 mM-EDTA. The treatment did not affect the adherence of the cells. Most of the radioactivity removed in the presence of EDTA was in the first two washes.

Ligand and immunoblotting

Blots for detecting the LDL receptor were performed as described (Windler *et al.*, 1988) using HepG2 cells scraped off the plates and homogenized by 5 strokes in a Dounce homogenizer. Receptor proteins were detected using biotinylated lipoproteins as described (Wade *et al.*, 1985), or by incubation of nitrocellulose membranes with antibodies directed against the LDL receptor (C7 and R48) at 4 °C for 12 h followed by incubation with an anti-mouse antibody [peroxidase-conjugated goat anti-mouse IgG (H+L); Jackson ImmunoResearch, Philadelphia, PA, U.S.A.] for 3 h at 24 °C. Blots for detecting the ASGP receptor used protein from microsomal membranes, prepared as described (Bischoff & Lodish, 1987) and separated on SDS/12%-polyacrylamide gels, and the monoclonal antibody 28 D6 against the ASGP receptor.

Analyses

Protein was determined according to the Lowry method, as modified for lipoproteins with BSA as standard (Sata *et al.*, 1972). Lipids were determined by standard procedures with reagents from Boehringer (Stähler *et al.*, 1977; Wahlefeld, 1974). Column chromatography (55 cm × 0.9 cm) was performed on Sephacryl CL-4B (Pharmacia), with Tris buffer (3 min/h; 1 ml fractions) at room temperature. All results are means of *n* experiments in duplicate or triplicate assays as indicated.

RESULTS

Binding of LDL and chylomicron remnants to the LDL receptor

Ligand blots of LDL and chylomicron remnants revealed binding to a protein in HepG2 cells with an apparent molecular mass of approx. 130 kDa, identified as the LDL receptor by immunoblotting with polyclonal and monoclonal antibodies (R48 and C7). There was no band of lower molecular mass (Fig. 1). Incubation of lipoproteins in Ca^{2+}-free buffer containing 10 mM-EDTA yielded no binding. Immunoblotting to identify the location of components of the ASGP receptor using the monoclonal antibody 28 D6 showed a major band with an apparent molecular mass of approx. 50 kDa, which was compatible with the H2 subunit of the human ASGP receptor as described previously (Shia & Lodish, 1989).

Specific cell association at 4 °C, representing binding, and specific cell association at 37 °C, representing binding and internalization, of [^{3}H]chylomicron remnants and ^{125}I-LDL by HepG2 cells were saturable (Fig. 2). Specific cell association, binding and internalization of ^{3}H labelled remnants were identical

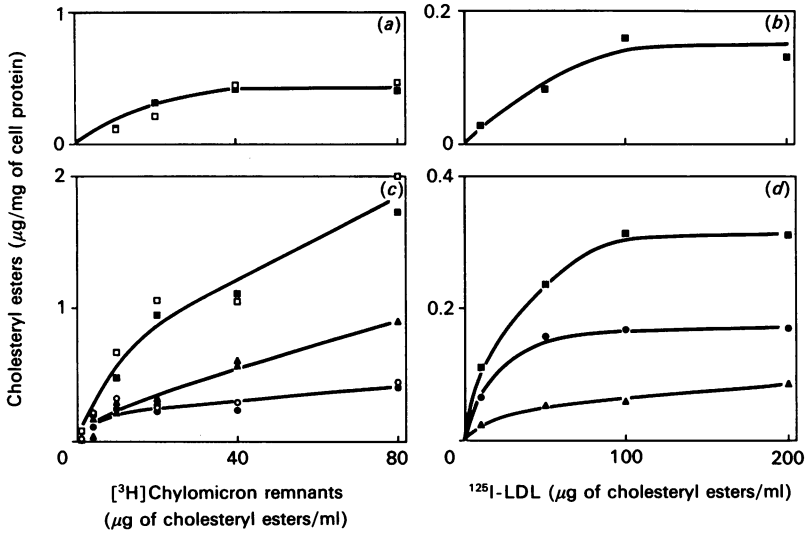

Fig. 2. Specific binding and internalization of [^{3}H]chylomicron remnants and ^{125}I-LDL by HepG2 cells

[^{3}H]Chylomicron remnants or ^{125}I-LDL were incubated with HepG2 cells seeded at 10^6 cells/dish at 4 °C or 37 °C for 1 h. In order to calculate specific binding, non-specific binding was determined by incubating ^{125}I-LDL in the presence of unlabelled LDL (5 mg of cholesteryl esters/ml). For remnants, non-specific binding was determined by addition of either unlabelled LDL (5 mg of cholesteryl esters/ml) (□, ○, △) or a 20-fold excess of unlabelled remnants (■, ●, ▲). Specific cell association at 4 °C (*a*,*b*) represents binding, while specific cell association (□, ■) at 37 °C (*c*,*d*) comprises binding (△, ▲) plus internalization (○, ●). Data are means of two experiments carried out in duplicate.

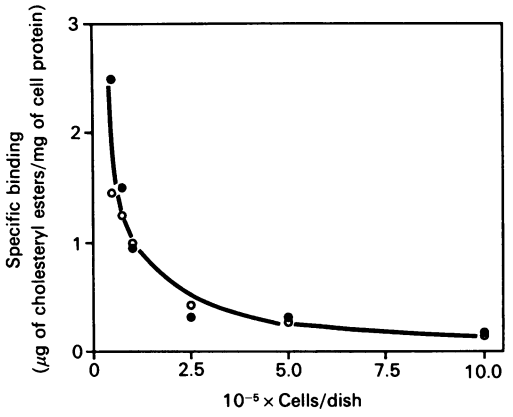

Fig. 3. Specific binding of ^{125}I-LDL and ^{125}I-chylomicron remnants to HepG2 cells at various cell densities

HepG2 cells were seeded at the indicated cell densities 48 h before the experiment. ^{125}I-LDL (●, 50 μg of cholesteryl esters/ml) or ^{125}I-chylomicron remnants (○, 10 μg of cholesteryl esters/ml) were incubated with the HepG2 cells at 37 °C for 1 h. Non-specific binding was determined by incubating the labelled lipoproteins in the presence of unlabelled LDL (3 mg of cholesteryl esters/ml). Results are given as means of duplicate assays.

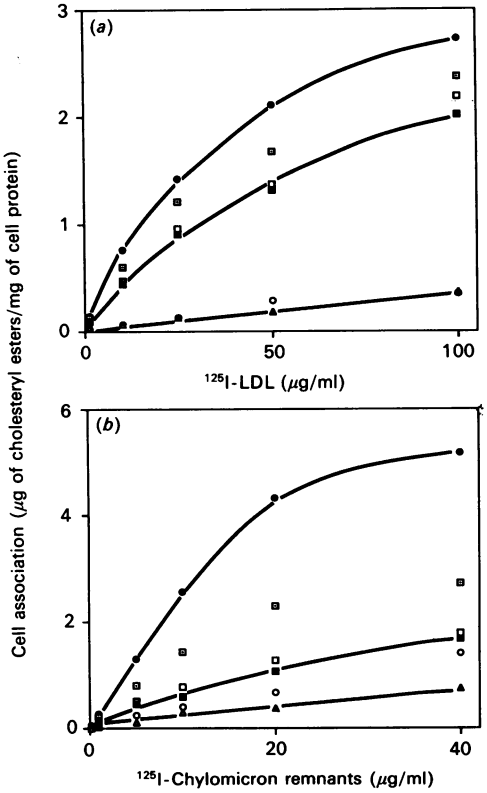

Fig. 4. Effect of asialofetuin on the association of (*a*) ^{125}I-LDL and (*b*) ^{125}I-chylomicron remnants with HepG2 cells

HepG2 cells were seeded at 5×10^5 cells per dish and incubated with ^{125}I-LDL ($n = 1$) or ^{125}I-chylomicron remnants ($n = 2$) at 37 °C for 1 h. For determination of the effects of excess LDL or asialofetuin, incubations were performed in the presence of LDL (3 mg of cholesteryl esters/ml) and various concentrations of asialofetuin. Results are given as means of n experiments carried out in duplicate assays. ●, Cell association; ○, +LDL; △, +LDL+asialofetuin (10 mg/ml). ⊡, +asialofetuin (1 mg/ml); □, +asialofetuin (10 mg/ml); ■, +asialofetuin (20 mg/ml).

Table 1. Effect of regulation of the LDL receptor on the specific binding of ^{125}I-chylomicron remnants to HepG2 cells

HepG2 cells were seeded at 5×10^5 cells per dish and grown for 2 days in a culture medium containing fetal calf serum and LDL (3 mg of cholesteryl esters/ml), or in a medium without fetal calf serum and without LDL, or in a medium supplemented with either 100 μM-17α-ethinyloestradiol, the HMG-CoA-reductase inhibitor pravastatin (200 μM), or both. ^{125}I-chylomicron remnants (40 μg of cholesteryl esters/ml) were incubated with the HepG2 cells at 37 °C for 1 h. Non-specific binding was determined by incubating ^{125}I-chylomicron remnants in the presence of excess unlabelled remnants (200 μg of cholesteryl esters/ml) or excess LDL (3 mg of cholesteryl esters/ml). Results of two experiments, carried out in duplicate, are expressed as percentages of the value obtained with cells grown in the presence of fetal calf serum and LDL.

	Specific binding (%)	
Culture medium	Excess remnants	Excess LDL
+LDL	100.0	117.6
−LDL	225.4	235.3
+17α-Ethinyloestradiol	420.2	440.5
+Pravastatin	468.2	485.6
+Oestradiol+pravastatin	569.1	690.4

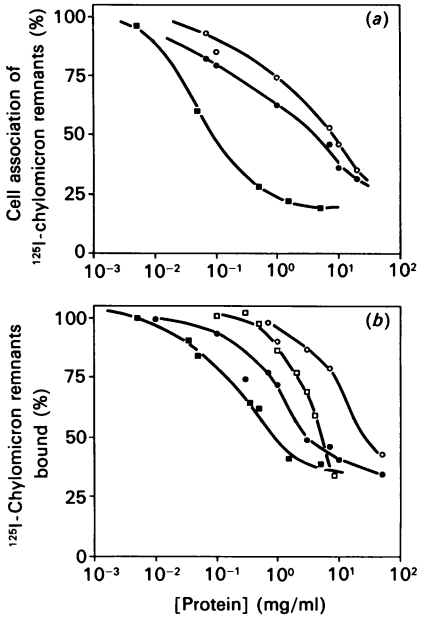

Fig. 5. Inhibition of the association of ^{125}I-chylomicron remnants with HepG2 cells by LDL, ASGPs and an antibody against the ASPG receptor

^{125}I-Chylomicron remnants (20 μg of cholesteryl esters/ml) were incubated with the HepG2 cells seeded at 10^6 cells per dish at (*a*) 37 °C ($n = 1$ each) or (*b*) 4 °C (n as specified) for 1 h. For competition studies incubations were performed in the presence of increasing concentrations of asialofetuin (●, $n = 3$), asialomucin (○, $n = 1$), LDL (■, $n = 2$) or hybridoma supernatant containing a monoclonal antibody directed against the ASGP receptor (□, $n = 2$). Results are given as means of n experiments carried out in duplicate.

whether determined by the addition of excess unlabelled LDL or excess remnants. The capacity for cell association and binding in terms of cholesteryl esters was higher for remnants than for LDL.

Specific binding of remnants was affected by measures known to regulate the LDL receptor in HepG2 cells (Leichtner *et al.*,

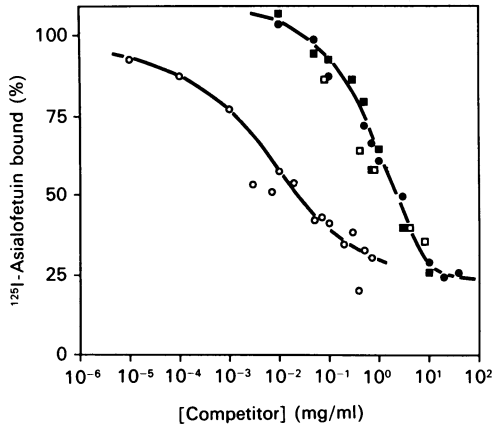

Fig. 6. Inhibition of the binding of ^{125}I-asialofetuin to HepG2 cells by asialofetuin, LDL, chylomicron remnants and an antibody against the ASGP receptor

^{125}I-Asialofetuin (6 μg of protein/ml) was incubated with HepG2 cells seeded at 10^6 cells per dish at 4 °C for 1 h. For competition studies, incubations were performed in the presence of increasing concentrations of asialofetuin (●, $n = 2$), a monoclonal antibody directed against the ASGP receptor (□, $n = 2$), LDL (■, $n = 2$) or chylomicron remnants (○, $n = 3$). Concentrations of competitors are expressed in terms of protein in the case of asialofetuin and the hybridoma supernatant containing antibody, and in terms of cholesteryl esters for LDL and chylomicron remnants. Results are given as means of n experiments carried out in duplicate assays.

Table 2. Inhibition of the binding of ^{125}I-asialofetuin, ^{125}I-LDL or ^{3}H-labelled chylomicron remnants to HepG2 cells by excess unlabelled ligands, an antibody or EDTA

Binding at 4 °C of ^{125}I-asialofetuin (6 μg of protein/ml), ^{125}I-LDL (100 μg of cholesteryl esters/ml), or [^{3}H]remnants (40 μg of cholesteryl esters/ml) to HepG2 cells was inhibited by excess asialofetuin (20 mg/ml) ($n = 2$), a monoclonal antibody directed against the ASGP receptor (8 μg of hybridoma supernatant protein/ml) ($n = 2$), LDL plus asialofetuin (3 mg of cholesteryl esters/ml plus 20 mg of protein/ml) ($n = 3$), chylomicron remnants plus asialofetuin (200 μg of cholesteryl esters/ml plus 20 mg of protein/ml) ($n = 1$) or 10 mM-EDTA ($n = 2$). Results are given as means of n experiments carried out in duplicate assays.

	Binding (%)		
	^{125}I-Asialofetuin	^{125}I-LDL	[^{3}H]Remnants
Ligand	100.0	100.0	100.0
+ Asialofetuin	22.0	71.5	70.0
+ Antibody	26.0	69.8	72.5
+ Unlabelled ligand + asialofetuin		33.0	37.2
+ EDTA	28.1	41.2	36.1

1984; Semenkovich & Ostlund, 1987). Binding of both ^{125}I-LDL and ^{125}I-chylomicron remnants greatly decreased with increasing cell density (Fig. 3). The capacity of binding of ^{125}I-chylomicron remnants to HepG2 cells was increased markedly by growing cells in the absence of LDL, and by supplementation of the medium with 17-α-ethinyloestradiol, the hydroxymethylglutaryl (HMG)-CoA-reductase inhibitor pravastatin, or both (Table 1). Specific binding was comparable whether determined by addition of either excess LDL or excess remnants.

Binding of LDL and chylomicron remnants to the ASGP receptor

Association of ^{125}I-LDL and ^{125}I-remnants to HepG2 cells at 37 °C was progressively lowered by addition of 1, 10 or 20 mg of asialofetuin/ml at all concentrations of the binding curve (Fig. 4). An asialofetuin concentration of 10–20 mg/ml apparently yielded maximal decreases of up to approx. 30% of the total specific cell association in the case of LDL and about 80% in the case of remnants.

At 37 °C, association of ^{125}I-remnants with HepG2 cells was efficiently decreased by addition of increasing concentrations of LDL, asialofetuin or asialomucin (Fig. 5). At 4 °C, binding of ^{125}I-remnants was affected by LDL and by these two ASGPs as well, and also by the monoclonal antibody 28 D6 directed against the ASGP receptor (Fig. 5). In this experiment, non-specific binding, determined by addition of an excess of unlabelled

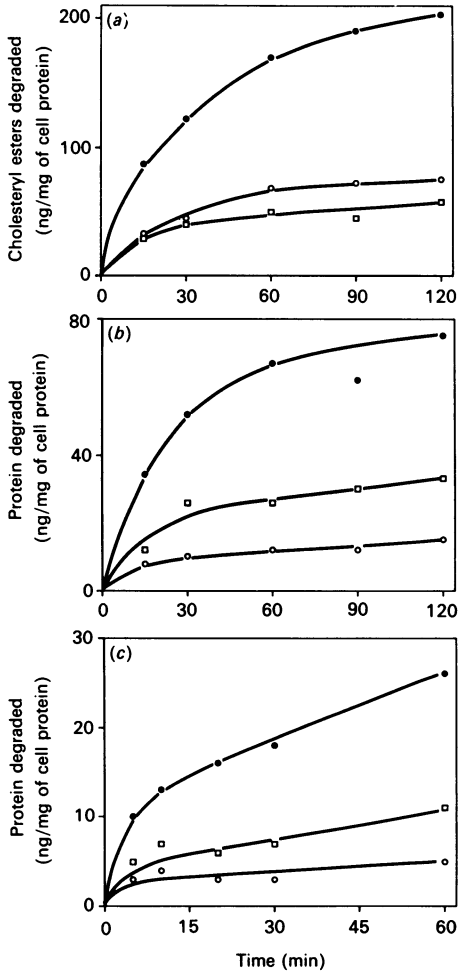

Fig. 7. Effects of asialofetuin, LDL and chylomicron remnants on the degradation of ^{125}I-chylomicron remnants and ^{125}I-asialofetuin by HepG2 cells

^{125}I-Chylomicron remnants (20 μg of cholesteryl esters/ml) (*a*) or ^{125}I-asialofetuin (6 μg/ml) (*b*,*c*) were incubated with HepG2 cells seeded at 10^6 cells per dish at 4 °C for 1 h. After four washes the cells were incubated at 37 °C, and at the indicated times non-protein-bound radioactivity was determined in the supernatant as a measure of degradation, expressed as ng of cholesteryl esters in ^{125}I-chylomicron remnants or ng of protein of ^{125}I-asialofetuin. (*a*) For determination of the effects of excess asialofetuin on LDL, the degradation of ^{125}I-chylomicron remnants (●) was determined by incubation in the presence of either LDL (□; 3 mg of cholesteryl esters/ml) or asialofetuin (○; 10 mg/ml). (*b*) The degradation of ^{125}I-asialofetuin (●) was determined by incubation in the presence of (*b*) either chylomicron remnants (□, 100 μg of cholesteryl esters/ml) or asialofetuin (○, 10 mg/ml) and (*c*) either LDL (□, 3 mg of cholesteryl esters/ml) or asialofetuin (○, 10 mg/ml). Experiments were carried out in duplicate.

Table 3. Effects of asialofetuin, LDL, or human remnants on the binding to human liver membranes of ^{125}I-LDL, [^{3}H]chylomicron remnants, ^{125}I-human remnants and ^{125}I-asialofetuin

^{125}I-LDL (100 μg of cholesteryl esters/ml) (n = 2), [^{3}H]chylomicron remnants (40 μg of cholesteryl esters/ml) (n = 2), ^{125}I-human remnants (20 μg of cholesteryl esters/ml) (n = 1) or 6 μg of ^{125}I-asialofetuin/ml (n = 1) were incubated with human liver membranes (1 mg of protein/ml) at 37 °C for 90 min. For competition studies incubations were performed in the presence of LDL (3 mg of cholesteryl esters/ml), asialofetuin (20 mg/ml), LDL plus asialofetuin (3 mg of cholesteryl esters/ml plus 20 mg of protein mg/ml), human remnants (200 μg of cholesteryl esters/ml) or human remnants plus asialofetuin (200 μg of cholesteryl esters/ml plus 20 mg of protein/ml). Results are given as means of n experiments carried out in triplicate.

	Binding (%)			
	^{125}I-LDL	[^{3}H]Remnants	^{125}I-Human remnants	^{125}-I-Asialofetuin
Ligand	100.0	100.0	100.0	100.0
+LDL	21.1	38.0	43.4	
+Asialofetuin	48.4	49.3	47.3	14.6
+LDL+asialofetuin	19.2	29.5	23.1	
+Human remnants				40.5
+Human remnants+asialofetuin				17.0

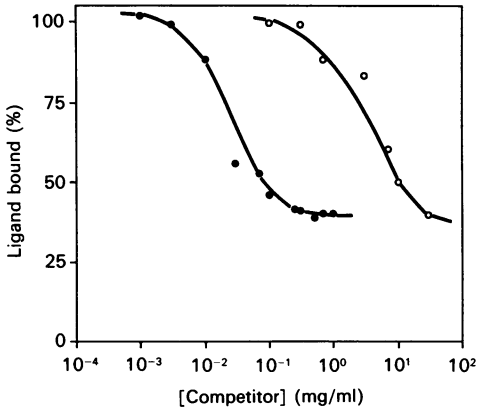

Fig. 8. Inhibition of the binding of ^{125}I-asialofetuin or ^{125}I-human remnants to HepG2 cells by excess human remnants or asialofetuin respectively

^{125}I-asialofetuin (6 μg/ml; n = 2) or ^{125}I-human remnants (20 μg of cholesteryl esters/ml; n = 1) were incubated with HepG2 cells seeded at 10^{6} cells per dish at 4 °C for 1 h. For competition studies incubations were performed in the presence of increasing concentrations of asialofetuin or human chylomicron remnants. Concentrations of competitors are expressed in terms of protein for asialofetuin and in terms of cholesteryl esters for remnants. Results are given as means of n experiments carried out in duplicate. ○, ^{125}I-human remnants+asialofetuin; ●, ^{125}I-asialofetuin+chylomicron remnants.

chylomicron remnants (1 mg of cholesteryl esters/ml), was 15.9%.

In cross-competition studies, the binding of ^{125}I-asialofetuin to HepG2 cells was efficiently competed for by asialofetuin itself, and by the antibody against the ASGP receptor (Fig. 6). LDL and remnants were also effective; however, remnants caused a 50% inhibition of binding at an approx. 100-fold lower concentration (in terms of cholesteryl esters in each lipoprotein) than did LDL.

The monoclonal antibody against the ASGP receptor caused inhibition of binding at 4 °C of ^{125}I-asialofetuin, ^{125}I-LDL or [^{3}H]remnants to HepG2 cells, which was comparable to the inhibition caused by excess asialofetuin (Table 2). Rinsing with a buffer containing 10 mM-EDTA abolished all of the specific binding of ^{125}I-asialofetuin. In the case of ^{125}I-LDL or [^{3}H]remnants, the effect of EDTA was comparable with that caused by the addition of excess asialofetuin plus LDL or remnants respectively, which exceeded the effect of excess asialofetuin alone. Two control antisera, hybridoma supernatant containing antibody raised against human apolipoprotein C-III or an IgG class 2 antibody raised against an unrelated antigen, used at the same protein concentration as the antibody against the ASGP receptor, had no effect on the binding of ^{125}I-asialofetuin (means of duplicate assays were 104% and 119% of the control value respectively).

In control experiments at 4 °C transferrin (10 mg/ml), the specific ligand of the transferrin receptor, decreased the total binding of ^{125}I-chylomicron remnants to HepG2 cells by no more than 2.8%, with no effect on ^{125}I-LDL binding (results not shown). Also, BSA at 1 or 10 mg/ml did not affect association to cells at 37 °C of ^{125}I-chylomicron remnants at five concentrations between 1 and 40 μg of cholesteryl esters/ml. BSA at 10 mg/ml did not affect the cell association of ^{125}I-LDL at five concentrations between 1 and 100 μg of cholesteryl esters/ml. Likewise, in two experiments at 4 °C in duplicate assays, 10 mg of human serum albumin/ml decreased the total binding of ^{125}I-chylomicron remnants by a mean of only 8%, as compared with 66% in the case of 10 mg of asialofetuin/ml in this experiment. However, galactosylated BSA at concentrations of 0.4, 0.8, 4 and 8 mg/ml lowered the total binding by 31, 36, 47 and 48%, as compared with 54% inhibition caused by asialofetuin at 7 mg/ml.

In order to demonstrate the functional significance of the inhibition of the binding and internalization of chylomicron remnants by ASGPs, pulse–chase experiments were performed. Internalization (results not shown) and degradation of ^{125}I-chylomicron remnants by HepG2 cells was inhibited by excess LDL as well as by excess asialofetuin (Fig. 7). LDL had a slightly greater effect than asialofetuin, but most of the degradation was also suppressible by addition of asialofetuin. Conversely, internalization (results not shown) and degradation of ^{125}I-asialofetuin was decreased by addition of excess LDL or remnants almost to the same extent as by addition of excess unlabelled asialofetuin itself (Fig. 7).

Binding of LDL and rat or human remnants to HepG2 cells or human liver membranes

Asialofetuin also competed with the binding of ^{125}I-LDL and [^{3}H]chylomicron remnants to human liver membranes at 37 °C, yet asialofetuin plus LDL had an even greater effect (Table 3). Comparable results were obtained using ^{125}I-labelled human

remnants, and in cross-competition studies human remnants were able to largely displace asialofetuin from its binding sites (Table 3). The antibody against the ASGP receptor decreased total binding of ^{125}I-human remnants to human liver membranes at 4 °C by 41 %, compared with 53 % in the case of added asialofetuin (20 mg/ml). The same protein concentration of hybridoma supernatant containing control antiserum against an unrelated antigen had no effect on the binding either of ^{125}I-asialofetuin or ^{125}I-human remnants (130 % or 118 % of control respectively; means of triplicate assays). Binding of ^{125}I-human remnants and ^{125}I-asialofetuin to HepG2 cells was successively decreased by rising concentrations of asialofetuin and human remnants respectively (Fig. 8).

In order to exclude the possibility that interaction of asialofetuin and lipoproteins in the cell medium possibly results in a non-specific decrease in the binding of lipoproteins to cell-surface receptors, remnants were incubated with asialofetuin at 4 °C for 2 h and re-isolated by column chromatography on Sepharose CL-4B. When ^{125}I-asialofetuin had been mixed with chylomicron remnants in ratios of 0.3, 1.5 and 5 (protein/cholesteryl esters, w/w) and were separated by chromatography, 1.95 %, 2.50 % and 0.56 % of the ^{125}I respectively was found in the region of the peak of the remnants, compared with 0.52 % in the case of ^{125}I-asialofetuin in the absence of remnants. When asialofetuin and remnants had been mixed in ratio of 5:1 (protein/cholesteryl esters, w/w), 97.6 % and 98.0 % of the triacylglycerols were eluted in the void volume, compared with 99.0 % and 99.1 % in the case of pure remnants. Thus there was no evidence for aggregation of remnants and asialofetuin, nor for destruction of remnants by asialofetuin.

DISCUSSION

The results of this investigation provide strong evidence that LDL and chylomicron remnants bind not only to the LDL receptor, but also to the human ASGP receptor. This is based on the following observations.

ASGPs such as asialofetuin or asialomucin and neoglycoproteins with terminal galactose residues, known to be specific ligands of the ASGP receptor (Geuze *et al.*, 1986), efficiently competed with the binding of chylomicron remnants and LDL. In order to exclude a non-specific effect at the LDL receptor, it was shown that, conversely, lipoproteins inhibit the binding of ASGPs. As a control for remnants labelled with ^{125}I, which is in part attached to possibly dissociable apolipoproteins and phospholipids, some experiments were performed using chylomicron remnants labelled with [^{3}H]cholesterol, of which more than 80 % represents cholesteryl esters in the core of the remnants (Windler *et al.*, 1988). Control experiments excluded an interaction of asialofetuin and lipoproteins within the incubation medium rather than at a cell-surface receptor. A strong additional indication for the specificity of the interaction of lipoproteins and the ASGP receptor is the inhibition of the binding of chylomicron remnants to HepG2 cells by the monoclonal antibody 28 D6 which is directed against the ASGP receptor. The effect was comparable with that in the binding of ASGPs. In the light of these observations, it also appears unlikely that the binding of lipoproteins to the LDL receptor is influenced by the interaction of ASGPs with the ASGP receptor due to closeness of the two receptors to each other.

The LDL receptor does not necessarily account for all of the specific binding that is not displaceable by excess ASGPs. The contributions of other binding sites, such as the LDL-receptor-related protein (Kowal *et al.*, 1989; Lund *et al.*, 1989), or even of other lectins, have to be considered. However, binding of chylomicron remnants to the LDL receptor in ligand blots and the effect of known stimuli for the LDL receptor activity on the binding of chylomicron remnants to HepG2 cells suggest a contribution of the LDL receptor to the hepatic uptake of chylomicron remnants. This is in line with previous experiments based on competition between remnants and LDL or an antibody directed against the LDL receptor and comparable observations using rat hepatocytes (Jensen *et al.*, 1987; Windler *et al.*, 1988; Jaeckle *et al.*, 1989). Moreover, the response of the LDL receptor to the requirements of the cell for cholesterol makes this receptor appear to be a metabolically important binding site. Based on the content of cholesteryl esters, chylomicron remnants displayed a much higher capacity of binding to HepG2 cells than did LDL. However that part of the total specific binding which presumably represents binding to the LDL receptor was comparable for LDL and chylomicron remnants. Thus the higher total binding capacity for remnants may be due to the interaction with the ASGP receptor. Previously, calculations from electron micrographs gave a content of cholesteryl esters in chylomicron remnants and LDL in the same order of magnitude, which accordingly roughly represents the number of particles (E. Windler & J. Greeve, unpublished work).

LDL bound with a much lower affinity to the ASGP receptor than did remnants. In an earlier investigation, desialylation of LDL did not lead to enhanced catabolism *in vivo* or by cultured cells. However, measurement of sialic acid and galactose residues suggested that the native LDL, which served as a control, was already substantially desialylated (Attie *et al.*, 1979). Also, binding to the LDL or ASGP receptor was not distinguished, so that the ASGP receptor may have played a role in these experiments. The ability of galactosyl residues to mediate uptake of LDL into hepatocytes and the possibility of the presence of asialo-LDL *in vivo* has been shown (Attie *et al.*, 1980; Bernini *et al.*, 1988; Orekhov *et al.*, 1989). Thus a contribution of the ASGP receptor to the hepatic uptake of LDL, especially in the absence of LDL receptors, is still conceivable.

In the competition experiments, remnants exhibited an at least 100-fold higher affinity to the ASGP receptor, than did LDL, based on their content of cholesteryl esters. Furthermore, the concentration of asialofetuin yielding 50 % competition with the specific binding of remnants is roughly 20-fold higher than that of the remnants on the basis of their protein content, suggesting a remarkably higher affinity of the remnants for the ASGP receptor compared with that for asialofetuin. This is also supported by the finding that a 10-fold higher concentration of antibody is necessary for inhibiting binding of remnants to the ASGP receptor to the same extent as inhibition of asialofetuin binding. Therefore the ASGP receptor may well have a physiological function in the uptake of remnants *in vivo*. The expression of the ASGP receptor on hepatocytes and its co-endocytosis with the LDL receptor is in line with the localization of remnant uptake formerly observed (Jones *et al.*, 1984; Belcher *et al.*, 1987). The proportions of the LDL and ASGP receptors, however, cannot reliably be estimated from results obtained in cell cultures. The activities of these receptors differ at low and high cell densities, since the LDL receptor is more active in growing cells and in the absence of exogenous cholesterol, while the ASGP receptor is primarily expressed on full-grown hepatocytes (Leichtner *et al.*, 1984; Steer *et al.*, 1987; Mazzone *et al.*, 1989). The standard assays in the present investigation used cells at high density grown for 24 h in lipoprotein-deficient medium. However, the possibility of a physiological function is supported by the demonstration of a substantial contribution of the ASGP receptor to the binding of remnants on membranes from human liver, which probably reflect more closely expression and activity of receptors *in vivo* than do cultured cells. Too-low receptor activity might have been the reason for the failure to disclose

binding of lipoproteins to the ASGP receptor in previous studies (Cooper & Coleman, 1985). This problem may also arise when using cells from species other than the rat, in which the LDL receptor probably plays a predominant role (Nagata *et al.*, 1988). Additional difficulties in the detection of the ASGP receptor can stem from the method of lipoprotein preparation, which may cause variation in the content of sialic acid and terminal galactose.

Furthermore, for the following reasons binding of lipoproteins to the ASGP receptor may easily be confused with binding to the LDL receptor. Because both LDL and chylomicron remnants, though in different proportions, are able to bind to the ASGP receptor, competition experiments using these two lipoproteins cannot distinguish between the LDL receptor and the ASGP receptor. Since remnants display a much higher affinity for the ASGP receptor than does LDL, which is similar to that of remnants for the LDL receptor, Scatchard plots may not reveal binding to two separate receptors. Ca^{2+}-dependence represents another characteristic feature of the ASGP receptor, but also of the LDL receptor. Finally, binding to the ASGP receptor may not be detected in ligand blots, since the assay conditions have previously been developed for the LDL receptor but not for the ASGP receptor, and the ASGP receptor may display its full activity only as a hetero-oligomer integrated in a membrane (Drickamer, 1987; Shia & Lodish, 1989; Braiterman *et al.*, 1989).

A contribution of the ASGP receptor to LDL and remnant clearance would provide the solution to various questions in lipoprotein metabolism. Though well quantified and largely localized to the liver, the uptake mechanism of the LDL-receptor-independent pathway is still unknown, and may well be partially mediated by the ASGP receptor (Goldstein & Brown, 1982; Pittman *et al.*, 1982; Edge *et al.*, 1986; Dietschy & Spady, 1986; Nenseter *et al.*, 1988). Under physiological conditions the ASGP receptor would allow the uptake of exogenous lipids in chylomicron remnants, even in states of down-regulated LDL receptor activity. In rat hepatocytes binding of remnants to sites distinct from the LDL receptor has previously been demonstrated (Nagata *et al.*, 1988). However, since this LDL-receptor-independent binding was not followed by internalization and did not require Ca^{2+}, it appears unlikely that it is identical with the binding to the ASGP receptor described for human liver cells in the present paper. Additionally, the ASGP receptor might in part be responsible for the clearance of LDL even in states of LDL receptor deficiency. The high affinity of remnants for the ASGP receptor may also explain the unimpeded turnover of remnants under these conditions. The concentrations of chylomicrons that are converted to remnants and thus are ready to be removed from the circulation at each instant may be low compared with the number of receptors, including the LDL receptor and alternative removal mechanisms. Therefore deficiency of one pathway as in familial hypercholesterolaemia may not have overt consequences under physiological conditions or in experiments using trace amounts of remnants. At saturating concentrations of remnants we could clearly demonstrate an influence of the LDL receptor activity on the rate of remnant removal in the rat (S. Jaeckle & E. Windler, unpublished work).

Clearly, the ultimate evaluation of the physiological role of the ASGP receptor will depend on experiments *in vivo*, e.g. competition studies, and demonstration of an effect of a modulation of receptor or ligand. However, it appears that the role of lectins in lipoprotein metabolism may have been previously underestimated.

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