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Characterization of the binding of human low-density lipoprotein to primary monolayer cultures of rat hepatocytes

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1. The binding of human low-density lipoprotein labelled with ^{125}I to rat hepatocytes in monolayer culture was measured at 4 °C. Evidence for two different specific binding sites was obtained. 2. Binding to Site 1 was characterized by: (*a*) being displaced by dextran sulphate or heparin; (*b*) being dependent on Ca^{2+}; (*c*) having a K_d value of about 15 μg of protein/ml; (*d*) not being significantly displaced by a 20-fold excess unlabelled low-density lipoprotein that had been reductively methylated; (*e*) being displaced by approx. 40% by a 20-fold protein excess of unlabelled human high-density lipoprotein, HDL_3, and (*f*) increasing with time in culture when newborn-calf serum was present in the medium. 3. The binding to Site 2 had the following properties: (*a*) it was not displaced by sulphated polysaccharides; (*b*) it was only partially Ca^{2+}-dependent, and the presence of EDTA increased the K_d value; (*c*) the apparent K_d value in the presence of Ca^{2+} was approx. 30 μg of protein/ml, which was significantly higher than for Site 1; (*d*) it was displaced by approx. 30% with a 20-fold excess of low-density lipoprotein that had been methylated; (*e*) it was displaced by unlabelled HDL_3 to a similar extent to Site 1; (*f*) it did not increase significantly with time in culture. 4. The characteristics of binding to Sites 1 and 2 are discussed in relation to the receptors for low-density lipoproteins that have previously been described in various cell types. 5. It is proposed that the experimental system described in this paper is suitable for studying the regulation of the binding of low-density lipoproteins to hepatocytes.

INTRODUCTION

The liver plays a central role in lipoprotein metabolism. As well as being the major site of synthesis of VLDL and HDL, it plays a part in the catabolism of chylomicrons, VLDL, LDL and HDL (for review see [1]). The catabolism of lipoproteins is thought to be mediated via specific cell-surface receptors, and distinct binding sites have been described for chylomicron remnants [2–3], LDL [4–6] and apo-E-free HDL [7–9]. LDL uptake appears, at least in part, to be mediated by a receptor similar to that described in a variety of cultured cell types by Goldstein & Brown (for review see [10]). As such, it is specific for apo-B and apo-E, and binding can be inhibited by modification of lysine or arginine of the apolipoprotein. Binding is Ca^{2+}-dependent, and LDL can be released from the receptor by the addition of sulphated polysaccharides such as heparin or dextran sulphate. Although the regulation of expression of such receptors has been extensively studied with cultured fibroblasts [10], little is known of such regulation in the liver, an organ which plays a specific role in the clearance of cholesterol from the circulation and in its excretion [11].

Specific binding sites for LDL other than the 'classic' LDL receptor have been described on isolated liver membranes and for hepatocytes in culture. In a study of human liver plasma membranes, Hoeg *et al.* [12,13] concluded that mature adult human liver metabolized LDL via a receptor genetically distinct from the classical LDL pathway. Binding to this site was characterized by only a partial dependence on Ca^{2+}, and was detected even on membranes obtained from patients suffering from homozygous familial hypercholesterolaemia. More recently Edge *et al.* [14] showed that monolayer cultures of hepatocytes from patients with familial hypercholesterolaemia appear to take up LDL by a mechanism distinct from the 'classic' LDL pathway. Similar findings have been reported for LDL binding [15] and metabolism [9] by primary monolayer cultures of pig hepatocytes. Ose *et al.* [16] have described Ca^{2+}-independent binding of rat LDL to freshly isolated rat hepatocytes, though binding of human LDL to such cells has been reported to be Ca^{2+}-dependent [17]. Both Ca^{2+}-sensitive and -insensitive LDL binding has been reported to hepatic plasma membranes [18] and to cultured hepatocytes [19] from rabbits.

The aims of the present study were to characterize the binding of human LDL to primary monolayer cultures of rat hepatocytes. These cells were shown to bind LDL specifically to two sites, one of which resembles the classic receptor described by Goldstein & Brown [10].

EXPERIMENTAL

Animals and materials

The sources of rats and most of the materials has been previously described [20,21]. ^{125}I was obtained from Amersham International, Amersham, Bucks., U.K. Dextran sulphate (average M_r 500000), heparin (sodium salt, grade 1) and hydroxypropylmethyl-cellulose (giving a viscosity of 50 cP in a 2% solution) and heparin–agarose were from Sigma (London) Chemical Co.

Preparation and incubation of hepatocytes

The preparation of hepatocytes from male Wistar rats (about 200 g) has been described [20]. The hepatocytes

Abbreviations used: apo-, apolipoprotein; HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very-low-density lipoprotein.

were attached, without using collagen, to Primaria tissue-culture dishes (Falcon) in a modified [22] Leibovitz L15 medium containing 10% (v/v) newborn-calf serum. Newborn-calf serum contained approx. 2 mM-cholesterol (as measured enzymically with a C-System Cholesterol Kit, from Boehringer, Mannheim, W. Germany) of which 62% was not precipitable by phosphotungstate and thus presumably associated with HDL [23]. In later experiments the medium used for bedding down the cells on to the culture dishes was supplemented with 0.6% (w/v) hydroxypropylmethyl-cellulose. This ensured a more even distribution of cells in the dishes [24], although this has not been necessary in earlier experiments [22]. The media were changed after 24 h, and binding experiments were performed after 48 h of culture, unless indicated to the contrary. Thus serum was present throughout the 48 h unless otherwise indicated.

Lipoprotein isolation and iodination

Human lipoproteins were prepared by sequential ultracentrifugation of freshly isolated plasma [25] taken from fasted healthy young males. LDL and HDL_3 were isolated within the density ranges of 1.020–1.060 and 1.125–1.210 g/ml respectively. Lipoproteins were washed once by ultracentrifugation and dialysed extensively against 0.15 M-NaCl containing 0.24 mM-EDTA, adjusted to pH 7.4 with NaOH. The protein concentration in lipoproteins was measured by Coomassie Brilliant Blue binding [26]. LDL was characterized by SDS/polyacrylamide-gel electrophoresis [27]. It contained only apo-B, with no detectable apo-A_1 or apo-E. The major apoprotein of HDL_3 was apo-A_1, and it was free of apo-B and apo-E.

Human LDL was iodinated with by the ICl method [28] as modified for lipoproteins by Shephard *et al.* [29]. Labelled LDL was separated from unbound iodine by passage through a column of QAE (quaternary aminoethyl)-Sephadex G-25 and by dialysis against 0.15 M-NaCl containing 0.24 mM-EDTA at pH 7.4. The final specific radioactivity of [^{125}I]LDL was 4×10^4–13×10^4 c.p.m./μg of protein, of which less than 3% was soluble in the presence of 5% (w/v) trichloroacetic acid.

To test whether ^{125}I-LDL was contaminated by apo-B-free but apo-E-containing lipoprotein particles, ^{125}I-LDL was purified on a heparin–agarose column as described by Weisgraber & Mahley [30]. No radioactivity or protein was eluted with 0.095 M-NaCl, suggesting that the preparation was essentially free of apo-E-containing particles.

Reductive methylation of the apo-B of LDL was carried out at 0 °C by addition of $NaBH_4$ and aqueous formaldehyde as described by Weisgraber *et al.* [31]. The extent of methylation was determined spectrophotometrically with trinitrobenzenesulphonic acid [32].

Lipoprotein-binding assay

The binding of [^{125}I]LDL to hepatocytes was determined by a modification of the procedure of Goldstein *et al.* [33]. After 48 h in culture, the dishes of hepatocytes were placed at 4 °C for 15 min. The medium was then replaced (unless stated to the contrary) with ice-cold Liebovitz L15 medium that was serum-free and which contained 2 g of fatty-acid-poor bovine serum albumin/l. The cells were then left at 4 °C for a further 15 min. Fresh albumin-containing medium was then added containing the specified amount of ^{125}I-LDL in the presence or absence of unlabelled lipoprotein. The cells were normally incubated for 4 h at 4 °C, and they were then rapidly washed with 3×2.5 ml of ice-cold 50 mM-Tris buffer adjusted to pH 7.4 with HCl, containing 0.15 M-NaCl and 2 g of albumin/l. Each monolayer was then incubated twice, for 10 min, with 2.5 ml of the same buffer, followed by three rapid washes with the Tris/NaCl buffer but without albumin. The plates were then treated with 2 ml portions of 10 mM-Hepes adjusted to pH 7.4 with NaOH and containing 50 mM-NaCl and 4 g of dextran sulphate/l, and then incubated for 1 h at 4 °C in a rotary shaker (60 rev./min). The dextran sulphate solution was then collected, and a 1.5 ml sample was taken to determine the amount of ^{125}I-LDL that was released. LDL released into

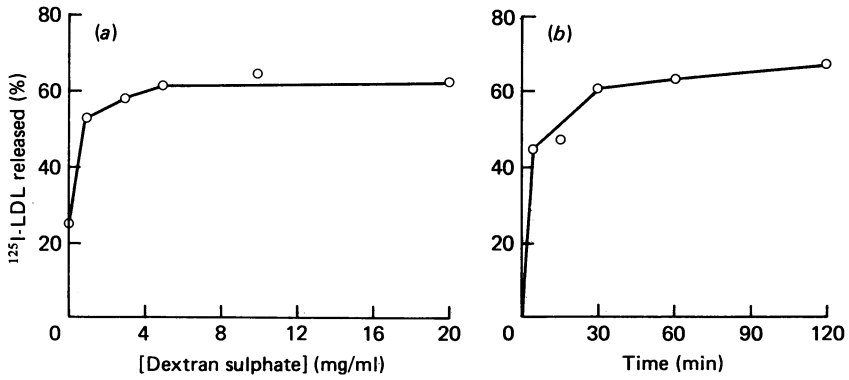

Fig. 1. Effect of dextran sulphate on the binding of ^{125}I-LDL to rat hepatocytes

Rat hepatocytes were maintained in monolayer culture for 48 h and then incubated with ^{125}I-LDL (10 μg of protein) at 4 °C for 4 h as described in the Experimental section. Fig. 1(*a*) shows the effect of a subsequent incubation for 1 h with various concentrations of dextran sulphate on the displacement of total ^{125}I-LDL bound to the hepatocytes. In Fig. 1(*b*) the time of the subsequent incubation with 4 mg of dextran sulphate/ml was varied. Similar curves were obtained in a further independent experiment with dextran sulphate and in three in which dextran sulphate was replaced by heparin.

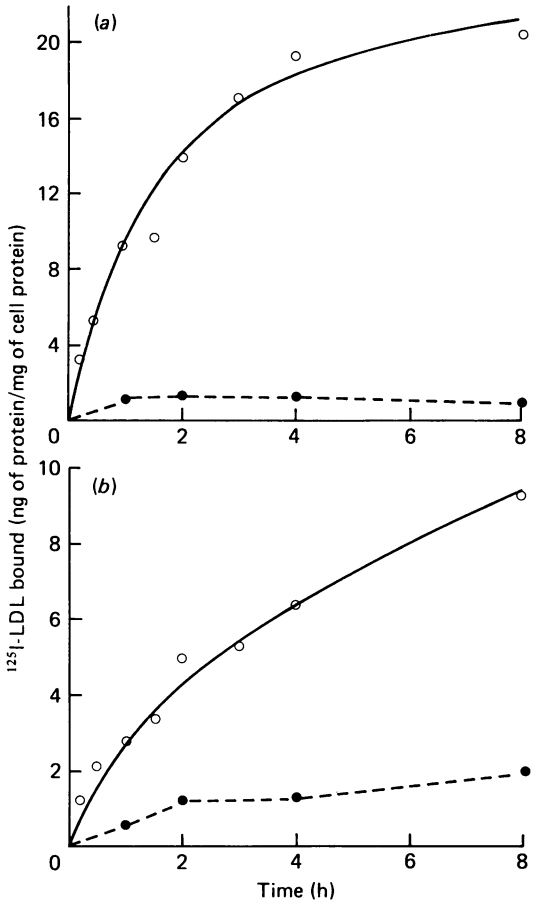

Fig. 2. Time course of the binding of ^{125}I-LDL to rat hepatocytes

Rat hepatocytes were maintained in monolayer culture for 48 h and then incubated at 4 °C with ^{125}I-LDL (10 μg of protein/ml) in the presence (●) or absence (○) of 500 μg of unlabelled LDL protein/ml for the times indicated as described in the Experimental section. Any binding in the presence of 500 μg of unlabelled LDL/ml was assumed to be non-specific. The binding was divided into that which could be displaced by a subsequent incubation for 1 h with 4 mg of dextran sulphate/ml (Fig. 2*a*) and that which could not (Fig. 2*b*). The binding in these two Figures is designated as being associated with Sites 1 and 2 respectively. Essentially identical time courses were reproduced in a subsequent independent experiment.

the washing buffer immediately before and after the dextran sulphate incubation represented less than 10% of that released by dextran sulphate. The cells were washed a further three times with the albumin-free Tris/NaCl buffer and then scraped from the plates in 1 ml of this buffer. The cells were then pelleted in a bench centrifuge at 4 °C and finally dissolved in 1 ml of 0.1 M-NaOH. A sample (0.75 ml) of this cell suspension was counted for radioactivity to determine the amount of radioactivity still associated with the cells. A further sample was used to determine the concentration of cell protein. Results are expressed as ng of ^{125}I-LDL protein bound/mg of cell protein. Non-specific binding was determined in the presence of 500 μg of unlabelled LDL protein/ml. Specific binding was calculated by subtracting non-specific from total binding. Results shown represent mean values of duplicate determinations.

RESULTS

Release of bound ^{125}I-LDL by dextran sulphate

After 48 h in culture, hepatocytes were cooled, washed and incubated with ^{125}I-LDL for 4 h at 4 °C. At the end of this incubation, plates were washed (see the Experimental section) and incubated with dextran sulphate. Fig. 1(*a*) shows that, under the conditions recommended by Goldstein *et al.* [34], i.e. 4 mg of dextran sulphate/ml for 60 min, only 60% of ^{125}I-LDL associated with the cells was released. Increasing the concentration of dextran sulphate (Fig. 1*a*) or the length of incubation (Fig. 1*b*) had little effect on the amount of ^{125}I-LDL released. Similar results were found when dextran sulphate was replaced with heparin (results not shown).

Fig. 2 shows the time course of ^{125}I-LDL binding. Non-specific binding was measured in the presence of 500 μg of unlabelled LDL protein/ml. Fig. 2(*a*) shows that the dextran-sulphate-releasable binding approaches equilibrium after 3–4 h and that at this point approx. 95% of the binding is specific. The major portion of binding that was not releasable by dextran sulphate (Fig. 2*b*) was also shown to be specific (approx. 85% at 4 h). However, even at 8 h, binding had not reached equilibrium. These findings suggest that ^{125}I-LDL may be binding to two distinct sites, one (Site 1) from which LDL may be released by treatment with sulphated polysaccharides and the other (Site 2) which is resistant to such treatment.

When ^{125}I-LDL had been purified by chromatography on heparin–agarose (see the Experimental section), it still bound to two distinct sites.

The concentration-dependent increase in ^{125}I-LDL binding to Site 1 and Site 2 is shown in Fig. 3. Total binding was measured at increasing concentrations of ^{125}I-LDL alone, and non-specific binding was measured in the presence of 500 μg of unlabelled LDL protein/ml. Specific binding was calculated by subtracting non-specific from total binding. For both Site 1 and Site 2, non-specific binding increased in proportion to the concentration of ^{125}I-LDL, but specific binding appeared to reach saturation. Analysis of specific binding by the method of Scatchard [35] yielded linear plots for both sites, suggesting that each consists of a single binding component. In five different experiments, the dissociation constants (K_d) for binding to Sites 1 and 2 were 15.2 ± 5.8 and 29.8 ± 12.4 μg of LDL protein/ml (Means ± S.D.) respectively. These were found to be significantly different ($P < 0.05$) by a paired t test. It should be noted that, since binding to Site 2 does not appear to reach equilibrium at 4 h, the K_d quoted can only be regarded as an approximate estimation of affinity.

Effects of EDTA and Ca^{2+} on binding of LDL

Omission of Ca^{2+} from the buffer used in the binding studies did not significantly affect the binding of ^{125}I-LDL to either Site 1 or Site 2 (Table 1). However, when as little as 0.1 mM-EDTA was added, specific binding to site 1 was almost completely abolished (Fig. 4*a*). In the presence of 1 mM-EDTA, binding to Site 1 could be recovered by the addition of $CaCl_2$ (Fig. 4*b*), suggesting an absolute requirement for Ca^{2+} in binding to Site 1. By contrast, binding to Site 2 was only partially affected by adding EDTA (Fig. 4*c*), and this change failed to reach statistical significance in the five experiments described in

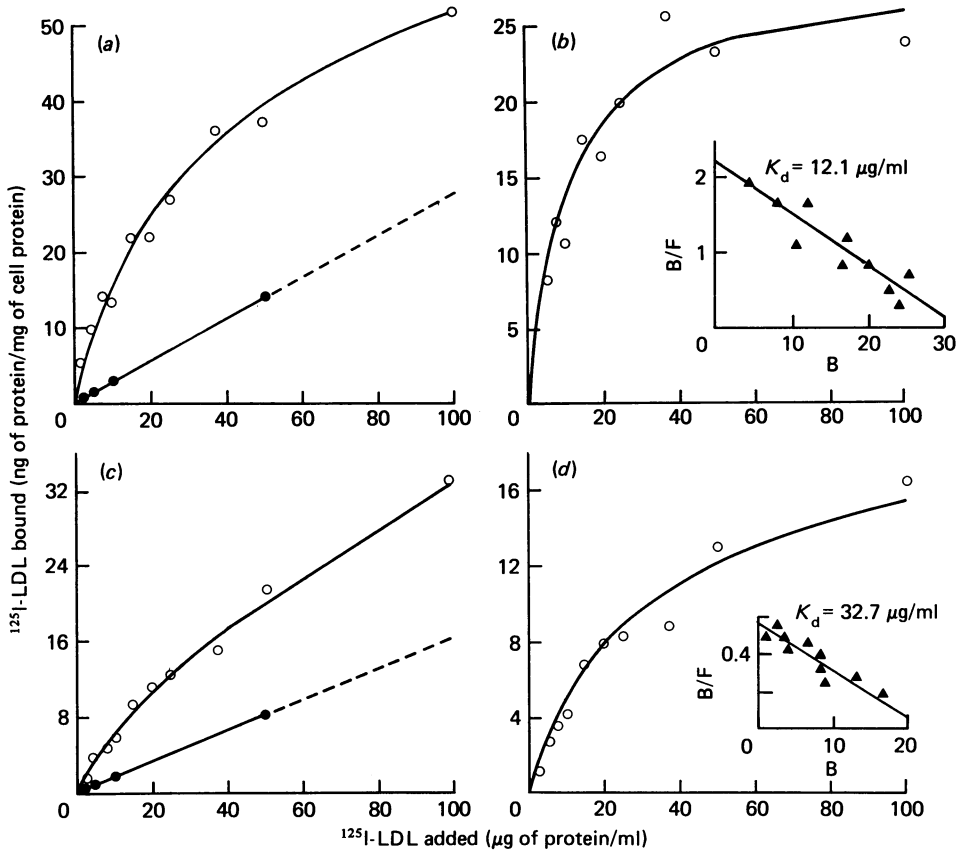

Fig. 3. Effect of the concentration of LDL on its binding to the dextran sulphate-releasable site (Site 1) and the non-releasable site (Site 2)

Rat hepatocytes were maintained for 48 h in monolayer culture and incubated at 4 °C for 4 h with various concentrations of ^{125}I-LDL in the presence or absence of 500 μg of unlabelled LDL protein/ml (see the Experimental section). The cells were then incubated for 1 h with 4 mg of dextran sulphate/ml. The amount of ^{125}I-LDL that was released is shown in Figs. 3(*a*) and 3(*b*), and that which remained bound is indicated in Figs. 3(*c*) and 3(*d*). Non-specific binding (●) is defined as the ^{125}I-LDL that was bound to the cells in the presence of 500 μg of unlabelled LDL protein/ml. To decrease the amount of unlabelled LDL required for each experiment, this was measured only at certain points and the line was extrapolated by linear-regression analysis. This was subtracted from the total ^{125}I-LDL bound (Figs. 3*a* and 3*c*) to give the appropriate values for specific binding (Figs. 3*b* and 3*d*). The insets show the analysis of the specific binding by the method of Scatchard [35], where B = bound LDL and F = free LDL. The results were reproduced in four further independent experiments.

Table 1. The addition of Ca^{2+} restored the binding of LDL to Site 2. The effect of EDTA was further examined in two experiments in which full concentration curves of ^{125}I-LDL binding in the presence of 1.26 mM-Ca^{2+} or 1 mM-EDTA were determined (Table 2). In both experiments both affinity and binding capacity of Site 1 were markedly decreased in the presence of EDTA. However, while K_d of binding to Site 2 was somewhat increaed, $B_{max.}$ changed little when EDTA was added.

Specificity of binding for LDL

The ability of HDL_3 to inhibit ^{125}I-LDL binding is shown in Fig. 5. Although HDL_3 did show some inhibition of binding, even at a 20-fold protein excess (equivalent to about a 120-fold excess of HDL particles), HDL_3 only inhibited binding to Sites 1 and 2 by $39 \pm 4\%$ and $36 \pm 20\%$ respectively in three independent experiments. However, this value is considerably higher than with human fibroblasts, in which at a 40-fold excess of HDL_3 protein from the same preparation only inhibited [^{125}I]LDL binding by 10% (A. M. Salter, J. Saxton & D. N. Brindley, unpublished work).

The specificity of LDL binding was further examined by using LDL in which lysine residues of apo-B were modified by reductive methylation. Fig. 6 shows that methylated LDL competes poorly with the native particle for binding to both Sites 1 and 2. Table 3 compares the ability of a 20-fold excess of native and methylated unlabelled LDL to compete with ^{125}I-LDL in four different experiments. Methylation decreased the ability of LDL to compete for binding to both sites in all experiments. However, the effect was generally greater on Site 1, where the binding of ^{125}I-LDL was actually stimulated in two of the four experiments.

Effect of time in culture on the expression of binding sites

Binding of ^{125}I-LDL was examined as a function of time in culture of hepatocytes (Table 4). Cells were maintained for the first 20 h in culture in the presence of 10% (v/v) newborn-calf serum. Some plates were taken to measure binding, and the medium on the others was changed to either fresh medium containing serum or serum-free medium supplemented with 2 g of bovine

Table 1. Effects of EDTA and Ca^{2+} on the binding of ^{125}I-LDL to rat hepatocytes

Rat hepatocytes were maintained in monolayer culture for 48 h and then incubated for 15 min at 4 °C. The Leibovitz L15 medium was then replaced with an ice-cold solution of 25 mM-Hepes, adjusted to pH 7.4 with KOH, containing 150 mM-NaCl and 2 g of fatty-acid-poor bovine serum albumin/l. The cells were then incubated for 15 min at 4 °C and the medium was replaced by fresh buffer containing Ca^{2+} or EDTA as indicated. ^{125}I-LDL (10 μg of protein/ml) was added and cells were incubated for 4 h at 4 °C. Cells were then treated with 4 mg of dextran sulphate/ml and incubated for a further 1 h. The ^{125}I-LDL released by dextran sulphate was defined as that bound to Site 1. ^{125}I-LDL that remained associated with the cells was defined as that bound to Site 2. Binding in the presence of 1.26 mM-Ca^{2+} is expressed as 100%. Results are expressed relative to this as means ± S.D. for five independent experiments.

Additions	Relative binding of LDL (%)	
	Site 1	Site 2
I Ca^{2+} (1.26 mM)	100	100
II No Ca^{2+}	95 ± 12	116 ± 25
III No Ca^{2+} + 5 mM-EDTA	23 ± 13	68 ± 22
I versus III, $P < 0.05$		

serum albumin/l. Binding was then measured 24 h later. In the presence of serum, binding to Site 1 significantly increased between 20 and 44 h. The removal of serum from the medium inhibited this increase. By contrast, binding to Site 2 did not significantly change in either the presence or the absence of serum between 20 and 44 h of culture.

DISCUSSION

Many studies of LDL metabolism by rat tissues have employed human rather than rat LDL, since rat plasma contains only low concentrations of LDL. It is also difficult to obtain it free from contamination by other lipoproteins. However, the kinetics of clearance of human and rat LDL from the serum of rats may be different [36,37]. In addition, cultured rat fibroblasts and smooth-muscle cells bind human and rat LDL differently [38,39]. Although the liver is the major site of LDL catabolism *in vivo* in the rat [40], hepatic plasma membranes of normal rats display little binding of human LDL that is saturable and of high affinity [4]. Furthermore, freshly isolated rat hepatocytes may not degrade human LDL [17]. It has therefore been suggested that the metabolism of human LDL does not reflect that of homologous lipoprotein in the rat.

By contrast, pharmacological doses of 17α-ethinyloestradiol caused a marked decrease in plasma cholesterol in the rat resulting from an up-regulation of LDL receptors in the liver [4,5]. Perfused livers from rats treated with 17α-ethinyloestradiol cleared significantly more rat or human LDL than did controls [41]. This was associated with an increased binding of human LDL to hepatic plasma membranes [4,5]. This binding shared many of the properties associated with LDL binding to its receptor on cultured fibroblasts: (1) it showed a marked preference for LDL as opposed to HDL, (2) it required Ca^{2+}, (3) it does not bind LDL in which lysine residues were methylated or acetylated, (4) binding was abolished by Pronase treatment of the membranes, and (5) binding was specific for apo-B and apo-E. The receptor that is increased by 17α-ethinyloestradiol from plasma membranes of rat liver migrated as a single protein with an apparent M_r of 133000 [42]. This compares with the M_r of 130000 found for the LDL receptor purified from bovine and rabbit adrenal cortex and human fibroblasts [43]. Thus it appears that rat liver can express specific high-affinity receptors that can bind and metabolize both human and rat LDL.

The present study demonstrates two specific binding sites for human LDL on rat hepatocytes in primary culture: one releasable by sulphated polysaccharides and the other resistant to such treatment. The release of LDL from its receptor by sulphated polysaccharides was originally described with cultured human fibroblasts [34]. Subsequent studies [44] showed that heparin interacts with positively charged guanido groups of arginine and ϵ-amino groups of lysine of apo-B and that these same residues are involved in cell-surface receptor binding of LDL. However, the specific nature of the interaction of apo-B with heparin and its receptor appears to be different, with the former being a purely ionic phenomenon but the latter is a more specific recognition of these two residues. The inability of dextran sulphate to release part of the LDL specifically bound to hepatocytes suggests either that arginine and lysine residues are not involved in the binding of LDL or that these residues are not accessible, when LDL is bound, to the dextran sulphate. This raises the possibility that LDL is binding to two different sites, Site 1, which is similar to the fibroblast receptor, and Site 2, representing a different interaction which in unaffected by sulphated polysaccharides.

Furthermore, Site 1 is similar to the receptor on fibroblasts [10] in that (1) it requires bivalent cations for activity, (2) it showed little affinity for HDL_3 and (3) it showed little affinity for LDL in which lysine residues had been modified by reductive methylation of apo-B. Similar characteristics have also been demonstrated for LDL binding to liver plasma membranes isolated from 17α-ethinyloestradiol-treated rats [4,5]. The mean K_d of 15.2 μg of LDL protein/ml agrees well with K_d values of 23, 18 and 17 μg/ml that have been reported for human LDL binding to liver plasma membranes isolated from 17α-ethinyloestradiol-treated rats [5], to solubilized receptors from liver plasma membranes of such rats [42] and to the purified receptor isolated from bovine adrenal cortex [45], respectively. This is somewhat higher than the values of 10.5, 8.2 and 6.6 μg/ml found for binding of homologous lipoprotein to pig hepatocytes [9], rat hepatoma H-35 cells [46] and liver plasma membranes taken from immature beagle dogs [2]. A much lower K_d of 3 μg of LDL protein/ml has been reported for binding of rat LDL to plasma membranes isolated from 1α-ethinyloestradiol-treated rats [5], and human LDL binds to human fibroblasts with a K_d of 2 μg/ml [10]. Thus it is possible that rat LDL would bind to rat hepatocytes with a greater affinity.

The fact that binding to Site 1 is up-regulated with time in culture (Table 4) may explain why studies on freshly isolated hepatocytes [7] have failed to demonstrate the

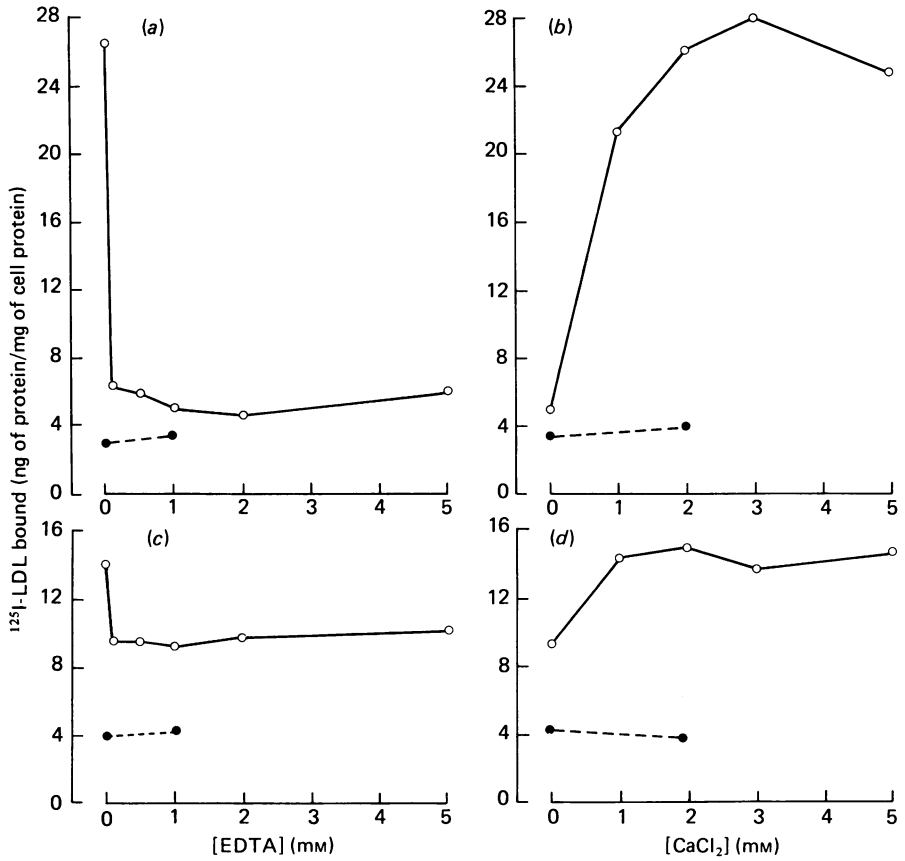

Fig. 4. Effects of EDTA and Ca^{2+} on the binding of ^{125}I-LDL to rat hepatocytes

Rat hepatocytes were maintained in monolayer culture for 48 h and then incubated for 15 min at 4 °C. The Leibovitz L15 medium was then replaced with an ice-cold solution of 25 mM-Hepes, adjusted to pH 7.4 with KOH, containing 150 mM-NaCl and 2 g of fatty-acid-poor bovine serum albumin/l. The cells were then incubated for 15 min at 4 °C, and the medium was replaced by fresh buffer containing EDTA and/or $CaCl_2$ as indicated. ^{125}I-LDL (10 μg of protein/ml) was added in the presence (●) or absence (○) of 500 μg of unlabelled LDL protein/ml respectively. The hepatocytes were incubated for 4 h at 4 °C. The cells were then treated with 4 mg of dextran sulphate/ml and incubated for a further 1 h. The ^{125}I-LDL that was released by dextran sulphate is shown in Figs. 4(*a*) and 4(*b*), and that which was not released is in Figs. 4(*c*) and 4(*d*). The full curve was reproduced in two further independent experiments.

Table 2. Effect of EDTA on the kinetics of ^{125}I-LDL binding to rat hepatocytes

Rat hepatocytes were maintained and washed as described in Table 1. Cells were then incubated with various concentrations of ^{125}I-LDL in the presence of either 1.26 mM-Ca^{2+} or 1 mM-EDTA for 4 h at 4 °C. Cells were then treated with dextran sulphate as described in Table 1. Non-specific binding was calculated as in Fig. 3 and was subtracted from total binding to give specific binding. Dissociation constant (K_d) and binding capacity ($B_{max.}$) were calculated by the method of Scatchard [35].

Expt.	LDL bound to	K_d (μg of LDL protein/ml)		$B_{max.}$ (ng of LDL protein/mg of cell protein)	
		1.26 mM-Ca^{2+}	1 mM-EDTA	1.26 mM-Ca^{2+}	1 mM-EDTA
I	Site 1	12.9	84.3	76	43
	Site 2	18.2	24.3	45	47
II	Site 1	9.6	49.8	28	8
	Site 2	15.6	31.0	16	14

presence of this type of binding site. Such regulation could represent either regeneration of receptors damaged after hepatocyte isolation or up-regulation by a component in the media. The absence of such an effect when serum is replaced by 2 g of fatty-acid-poor bovine serum albumin/l suggests the latter. HDL in the serum could act as a cholesterol acceptor and remove cholesterol from the cell, as in fibroblasts [47,48], and thus increase LDL uptake. Such a process has been described in the human heptoma line HepG2 [49]. The

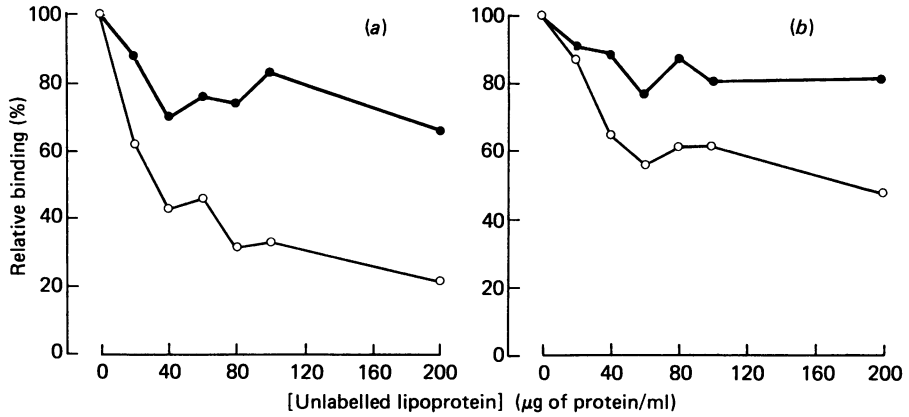

Fig. 5. Effects of unlabelled LDL and HDL_3 on the binding of ^{125}I-LDL to rat hepatocytes

Rat hepatocytes were maintained in culture for 48 h and then incubated for 4 h at 4 °C with ^{125}I-LDL (10 μg of protein/ml) and unlabelled LDL (○) or HDL_3 (●) at the concentrations indicated. This was followed by incubation for 1 h with 4 mg of dextran sulphate/ml. The relative amount of ^{125}I-LDL that was released is shown in Fig. 5(*a*) and that which was not released is in Fig. 5(*b*). Results were confirmed in two further experiments (see the text).

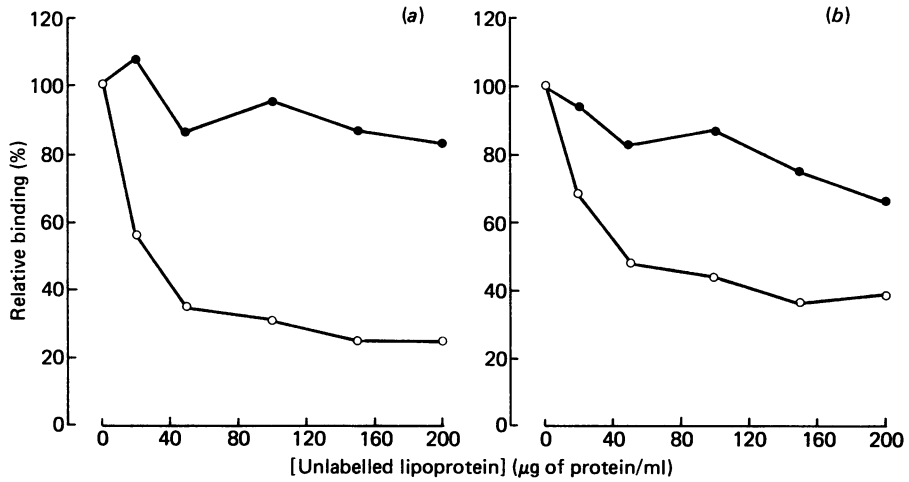

Fig. 6. Effects of methylated LDL on the binding of ^{125}I-LDL to rat hepatocytes

Rat hepatocytes were incubated with ^{125}I-LDL as in Fig. 5, but in the presence or absence of native LDL (○) or methylated LDL (●). Figs. 6(*a*) and 6(*b*) show the ^{125}I-LDL that was released or not released respectively by dextran sulphate. The results were essentially confirmed in four independent experiments (see Table 3).

newborn-calf serum that was used contained 62% of total cholesterol in the form of HDL. Furthermore, we now know that HDL_3 up-regulates Site 1, but not Site 2, whereas HDL_2 and LDL are without significant effect. This agrees with work with HepG2 cells [49] and suggests that the regulation of LDL binding to hepatocytes differs from that in other cells [10]. The different responses of Sites 1 and 2 in culture further confirm their separate identities.

Binding to Site 2, which is not displaced by sulphated polysaccharides, shows severeal other characteristics that are different from those to Site 1. Whereas the presence of EDTA effectively abolished binding to Site 1, the effect on Site 2 was much more variable (Table 1). On average, binding was inhibited by 45% in the presence of 1 mM-EDTA, compared with 87% for Site 1. Kinetic analysis of binding in the presence of EDTA shows that, although both affinity and binding capacity are markedly changed for Site 1, the major effect on site 2 is a decrease in affinity. It is possible that LDL measured as bound on Site 2 may be contaminated with LDL bound to Site 1. However, since increasing dextran sulphate up to 5 times the recommended amount [34] had no significant effect on the amount of LDL released, this seems unlikely. Ca^{2+}-independent binding of LDL has also been described in other work [5,12,18].

Site 2 exhibited similar specificity to Site 1 with respect to the recognition of both human HDL_3 and LDL in which apo-B lysine residues had been methylated. Whereas methylation did have some effect on the ability of LDL to compete with the native particle, this was highly variable and the overall effect on Site 2 was significantly less than on Site 1 (Table 3). It is surprising that sulphated polysaccharides, which interact with arginine and lysine residues, do not release LDL from Site 2, whereas modification of lysine residues does inhibit binding. One possible explanation is that methylation may result in a change in conformation of

Table 3. Effects of native and methylated unlabelled LDL on the binding of ^{125}I-LDL to rat hepatocytes in monolayer culture

Rat hepatocytes were maintained in culture for 48 h and then incubated with ^{125}I-LDL (10 μg of protein/ml) at 4 °C for 4 h in the presence of unlabelled native or methylated LDL as indicated as described in the Experimental section. The cells were then incubated for 1 h with 4 mg of dextran sulphate/ml. The amount of ^{125}I-LDL released was defined as that bound to Site 1. ^{125}I-LDL that remained associated with the cells was defined as that bound to Site 2. Results are means ± S.D. for four independent experiments, and that the significance of the differences between groups is shown by: $*P < 0.05$; $**P < 0.01$; $\dagger P < 0.001$.

	Additions	Binding (ng of LDL protein/mg of cell protein) Site 1	Site 2
I	None	16.4 ± 4.9	8.1 ± 0.8
II	+20-fold excess of LDL	3.8 ± 1.5	2.9 ± 0.7
		I versus II**	I versus II†
III	+20-fold excess of methylated LDL	16.6 ± 6.9	5.9 ± 1.9 I versus III*

Table 4. Effect of incubation time and of newborn-calf serum on the binding of ^{125}I-LDL to rat hepatocytes

Rat hepatocytes were maintained in monolayer culture as indicated. They were then incubated with ^{125}I-LDL (10 μg of protein/ml) at 4 °C for 4 h as described in the Experimental section. The cells were then incubated for 1 h with 4 mg of dextran sulphate/ml. The amount of LDL released by dextran sulphate as defined as that bound to Site 1 and that retained by the cells as Site 2. Specific binding was calculated by using results from parallel incubations in which 500 μg of unlabelled LDL protein/ml was used.

	Culture conditions	Specific binding (ng of LDL protein/mg of cell protein) Site 1	Site 2
I	24 h in 10% serum	6.2 ± 3.3	5.7 ± 0.5
II	48 h in 10% serum	12.1 ± 3.9 I versus II, $P < 0.05$	6.3 ± 2.3
III	24 h in 10% serum + 24 h in 0.2% albumin	7.7 ± 2.7	5.6 ± 1.4

the apo-B which may affect the molecular domain recognized by the binding site.

It is possible that contamination of our LDL fractions with apo-E could have modified binding to Site 1 (presumably an apo-B/E receptor) and to Site 2. However apo-E was not detected after SDS/polyacrylamide-gel electrophoresis in many preparations, and where it was the gels were grossly overloaded. Furthermore, no apo-B-free but apo-E-containing lipoprotein was isolated after chromatography of ^{125}I-LDL on heparin–agarose, and the LDL was eluted still bound to Sites 1 and 2.

The present study therefore demonstrates specific binding sites for human ^{125}I-LDL on rat hepatocytes in monolayer culture. Site 1 appears analogous to the LDL receptor previously described on cultured fibroblasts and a number of other systems and which is involved in receptor-mediated endocytosis. Site 2 exhibits significantly different characteristics to Site 1, and its physiological role, if any, remains to be identified. We do, however, know that the rate of LDL degradation by hepatocytes at 37 °C increases when binding to Site 1 is increased by culturing the cell with HDL_3. There also appears to be some residual degradation that is estimated to occur when the binding to Site 1 is extrapolated to zero (A. M. Salter, J. Saxton & D. N. Brindley, unpublished work). This may result from binding to Site 2. It is possible that this Site could account for the uptake of LDL by hepatocytes cultured from patients with familial hypercholesterolaemia [14]. Our monolayer culture system provides a useful model for further study of the regulation of the binding and metabolism of LDL by the liver. Such work could provide information as to how plasma cholesterol concentrations and the excretion of cholesterol from the body might be controlled and how we might intervene to lessen the risk of premature atherosclerosis.

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