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Characterization and specificity of lipoprotein binding to term human placental membranes

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The binding characteristics of very-low-density (VLDL), low-density (LDL) and high-density (HDL) lipoprotein fractions to a purified human term placental microvillous membrane preparation were determined. Binding of LDL was saturable with a maximal binding capacity of 270 ng LDL protein per mg of membrane protein. Scatchard analysis revealed the presence of a single population of $3.4 \cdot 10^{11}$ sites per mg of membrane protein and a mean affinity constant of $5.8 \cdot 10^{-9}$ M. Binding of VLDL was also saturable but the maximal capacity was 4.5-times greater than that of LDL. The Scatchard analysis revealed the presence of $2.1 \cdot 10^{11}$ binding sites and an affinity constant nearly one order of magnitude greater than that of LDL. Binding of HDL showed less tendency to saturate. Scatchard analysis showed a similar number of receptor sites to that calculated for VLDL and LDL but the affinity constant for HDL was over 100-fold less than that of VLDL. Self- and cross-inhibition studies of VLDL and LDL binding revealed that VLDL was better at blocking the binding of LDL than was LDL itself. This preferential binding of VLDL suggests that this lipoprotein fraction could be an important source of cholesterol for placental progesterone production.

Introduction

The human placenta at term produces about 250 mg of progesterone per day during the third trimester [1,2] but is unable to synthesise the necessary amount of precursor cholesterol for 2-carbon units [3]. Hence progesterone formed by the placenta must be derived from utilization of cholesterol in the maternal circulation [4]. Recent studies have shown that steroidogenic tissues possess two distinct mechanisms for uptake of lipoprotein cholesterol (for review, see Ref. 5). One process is specific for the apo B- and apo E-containing lipoproteins and involves receptor-mediated endocytosis followed by lysosomal degradation of the internalized ligands. This is the classical low-density-lipoprotein (LDL) pathway described by Goldstein and Brown [6] in fibroblasts. The second pathway involving high-density lipoprotein (HDL) is less well characterized.

A receptor on human placental microvillous membranes which binds LDL has been described [7,8]. These receptors were shown to be present from as early as six weeks gestation [9]. A mixed cell culture prepared from minced placental tissue was able to produce small quantities of pro-

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gesterone after LDL was added to a lipoprotein-deficient culture medium, presumably via these receptors [10]. In addition, a separate high-affinity membrane receptor for acetylated LDL has also been demonstrated [11].

Recent studies on human skin fibroblasts have shown that normal very-low-density lipoprotein (VLDL) binds almost as efficiently as LDL to the receptors, probably via apolipoprotein E [12]. During human gestation there is a steady increase in the maternal plasma cholesterol level, but, while the cholesterol content of LDL increases only marginally, there is a 3-fold increase in VLDL cholesterol [13]. We have therefore examined the binding of VLDL, LDL and HDL to a purified microvillous membrane preparation isolated from normal human term placenta using both self- and cross-inhibition studies. The data suggest that VLDL is preferentially bound to the lipoprotein receptor and therefore may be the preferential source of cholesterol for trophoblast in vivo.

A preliminary account of part of this study has been presented [14].

Materials and Methods

Placental microvillous membrane preparation

A syncytiotrophoblast microvillous membrane preparation was obtained as described by Contractor et al. [15]. Each preparation contained pooled membranes from 3–5 placentas. Briefly, normal term human placentas were collected immediately after vaginal delivery and placed in polythene bags on ice. Pieces of villous tissue were removed and placed in ice-cold 0.9% (w/v) saline. The tissue was rinsed to remove excess blood, gently blotted, and then scraped to remove the trophoblastic tissue. This was transferred to fresh ice-cold saline and gently stirred for 30 min at 4° C. A membrane pellet was prepared using differential centrifugation [16] which was 24-times enriched with the membrane marker enzyme alkaline phosphatase, as compared to a placental homogenate. This pellet was further purified by discontinuous sucrose gradient centrifugation [17] and the membranes which collected at the interface of the 25% and 37% (w/v) sucrose solutions were removed and washed with 10 mM Tris-HCl buffer (pH 7.3) containing 1 mM phenylmethylsulphonyl fluoride and 0.02% sodium azide. These membranes, which were now 35-times enriched with alkaline phosphatase, were stored at 4° C and used within a month of preparation.

Preparation of lipoprotein fractions

Blood was obtained from non-pregnant normolipidaemic individuals and collected into vials containing EDTA at a final concentration of 1 $mg \cdot ml^{-1}$. Plasma was separated by low-speed centrifugation and its density raised to 1.3 $g \cdot ml^{-1}$ by the addition of solid NaBr [18]. Lipoproteins were separated by rate zonal ultracentrifugation in a titanium B14 rotor on a Superspeed 65 Mark II ultracentrifuge (MSE Scientific Instruments, Crawley, Sussex) using a modification of the method of Patsch et al. [19]. A linear gradient was produced in the density range of 1.00–1.30 $g \cdot ml^{-1}$ and the plasma (about 100 ml) was added to the rotor. Separation of VLDL and LDL was achieved by centrifugation for 140 min at 42000 rpm. The rotor was unloaded in 10 ml fractions and the protein concentration was determined by absorbance at 280 nm. Representative peak samples of VLDL and LDL were concentrated by recentrifugation for 16–20 h at 28000 rpm in a 6×14 swinging bucket rotor. Lipoproteins were collected (by slicing the tubes) and dialysed into storage buffer containing 0.34 mM EDTA [20]. Purity of the VLDL (density < 1.006) and LDL (density 1.02–1.05) fractions was confirmed by agarose gel and SDS electrophoresis. Lipoproteins were iodinated using the iodine monochloride method to a specific activity of 300–400 $cpm \cdot ng^{-1}$ [20].

HDL was purified separately using a gradient ranging in density from 1.00–1.40 $g \cdot ml^{-1}$. In this case centrifugation was at 42000 rpm for 24 h. Representative peak samples of density > 1.063 were concentrated, characterized, and iodinated as described above.

Lipoprotein binding studies

The labelled lipoproteins were chromatographed just before use on a Sephadex G 25 column to remove any free iodine or small breakdown products. The protein concentration of the radioactive lipoprotein recovered in the void volume was determined [21] and the lipoprotein

was diluted with incubation buffer (100 mM NaCl, 10 mM Tris-HCl (pH 7.4) containing 1 mM $CaCl_2$ and 2% delipidated bovine serum albumin) to a concentration of 5 $\mu g \cdot ml^{-1}$. Binding studies were carried out in tubes containing 125 μg of microvillous membrane protein and a quantity of labelled lipoprotein between 300 ng and 1.5 μg. 300 μg of unlabelled lipoprotein was added to those tubes used to measure the amount of non-specific binding. All incubation volumes were 360 μl. Measurements were performed in duplicate and samples were incubated for 1 h at 4° C. Membrane pellets were obtained by centrifugation for 15 min and were washed once with 400 μl of incubation buffer and recentrifuged. The supernatants were combined. Binding of VLDL, LDL and HDL was determined by the method of Scatchard [22].

In the self- and cross-inhibition studies, tubes contained a constant amount of labelled lipoprotein (1.5 μg) and membrane (125 μg) and different amounts of either the same or a different unlabelled lipoprotein. Conditions were as described above. The effect of the unlabelled lipoprotein was quantified by expressing the amount of binding in its presence as a percentage of that measured in the absence of any competitor.

Results

Binding of lipoprotein subclasses

Fig. 1a shows the specific and non-specific binding curves obtained by incubating increasing amounts of ^{125}I-VLDL with 125 μg of microvillous membrane protein. The total binding data (not shown), obtained in the absence of excess unlabelled VLDL has been corrected for the non-specific binding measured in the presence of a 200-fold excess of VLDL, and this is plotted as the specific binding. Data are presented as mean ± S.E. from three separate experiments in which each value was the mean of duplicate determinations. A saturation curve weighted for the reciprocal of the standard errors of mean [23] has been fitted through the specific binding data. The calculated maximal binding capacity is 1215 ± 105 ng VLDL protein per mg of membrane protein. In Fig. 1b the individual values from the three assays are combined in a Scatchard plot. The linearity of the calculated regression line ($r = 0.70$, $P < 0.001$)

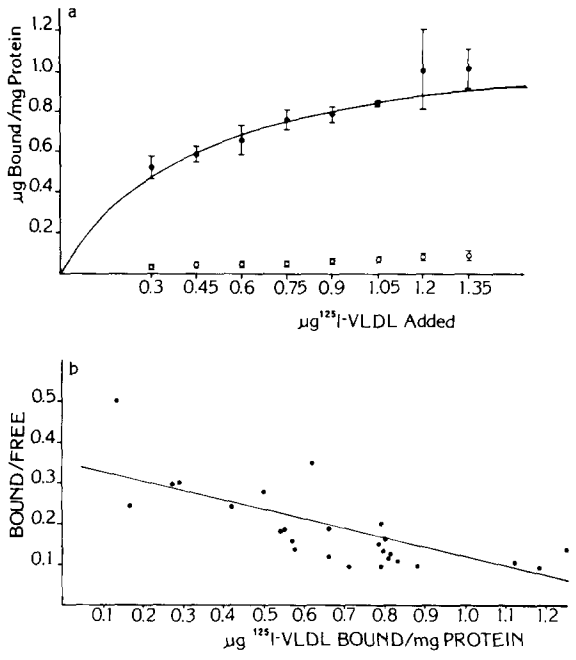

Fig. 1. (a) The specific (●) and non-specific (○) binding of ^{125}I-VLDL to human placental microvillous membranes. The amount of binding per mg of membrane protein is plotted against the amount of labelled lipoprotein present in the incubation. Experimental conditions are as described in the text. Values are plotted as mean ± S.E., $n = 3$ separate assays. The line fitted through the specific binding data is a weighted best fit calculated as described in the Results. (b) Scatchard analysis of the binding of VLDL to placental microvillous membranes. The 26 data points from the three assays are plotted individually and subjected to a linear regression. The correlation coefficient for the line ($r = 0.70$) is highly significant ($P < 0.001$). The intercept on the x-axis was used to calculate the number of binding sites present while the slope of the line was used to calculate the receptor affinity constant. Values are given in the text.

indicates a single class of binding sites. Assuming a mean protein content of VLDL of 5% and a relative molecular mass of $1 \cdot 10^8$ [18] it can be calculated that there are $2.1 \cdot 10^{11}$ binding sites per mg of membrane protein and that the mean affinity constant for the binding is $3.5 \cdot 10^{-10}$ M.

Fig. 2a illustrates the specific and nonspecific binding curves for LDL obtained in a similar manner to that described above except that the data are from four assays. The curve fitted through the specific data indicated a maximal binding capacity of 270 ± 16 ng of LDL protein bound per mg of membrane protein which is 4.5-fold less

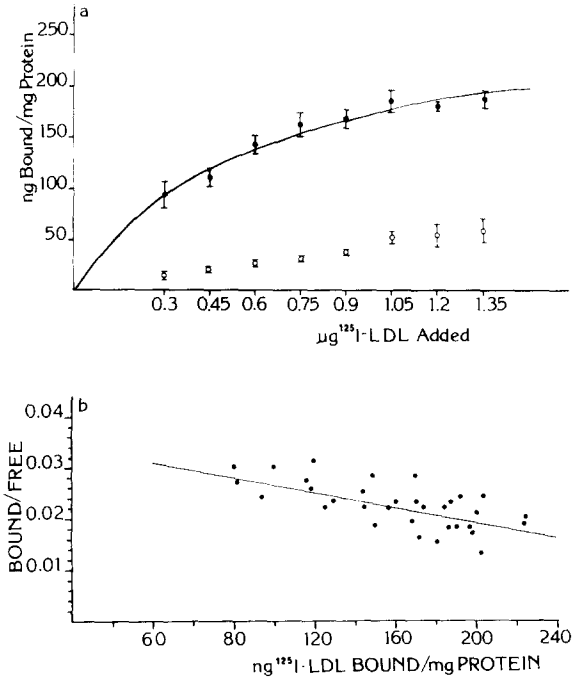

Fig. 2. (a) The specific (●) and non-specific (○) binding of ^{125}I-LDL to human placental microvillous membranes. Values are plotted as mean ± S.E., $n = 4$ assays. Details are given in the text. (b) Scatchard analysis of LDL binding data using 33 individual points from four assays. Details are as in Fig. 1 and in the text.

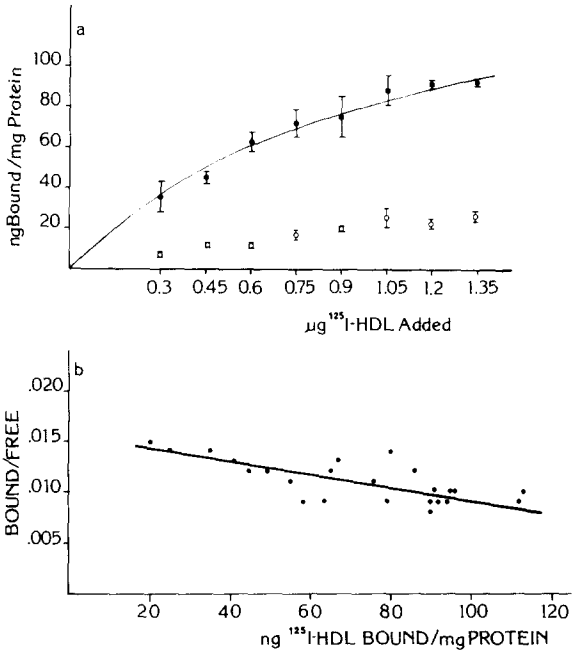

Fig. 3. (a) Specific (●) and non-specific (○) binding of ^{125}I-HDL to placental microvillous membranes. Values are plotted as mean ± S.E., $n = 3$ assays. Details are given in the text. (b) Scatchard analysis of HDL binding data using 25 individual points from the three assays. Details are as in Fig. 1 and in the text.

than that of VLDL. The Scatchard analysis (Fig. 2b) is also linear ($r = 0.70$, $P < 0.001$), and using a relative molecular mass for LDL of $3 \cdot 10^6$ and a protein content of 21% it can be calculated that there are $3.4 \cdot 10^{11}$ receptors per mg of membrane protein with the affinity constant being $5.8 \cdot 10^{-9}$ M.

Results for the specific and non-specific binding data for HDL are shown in Fig. 3a. The specific binding shows less tendency to saturate than was seen with VLDL or LDL. However, the fitted curve indicates a maximal binding of 178 ± 18 ng of HDL bound per mg of membrane protein, lower even than that found for LDL. The amount of HDL needed to achieve half maximal saturation is nearly twice that for LDL. Using a mean protein content of HDL of 50% and a relative molecular mass of $3.5 \cdot 10^5$ the Scatchard analysis (Fig. 3b) indicates a single class of receptors ($r = 0.67$, $P < 0.001$) accounting for $5.5 \cdot 10^{11}$ sites per mg of membrane protein with an affinity constant of $3.3 \cdot 10^{-8}$ M.

Self- and cross-inhibition of VLDL and LDL binding

The specificity of the binding of VLDL and LDL to the placental microvillous membrane preparation was examined by determining the effect of increasing concentrations of unlabelled VLDL or LDL on the binding of either ^{125}I-VLDL or ^{125}I-LDL. The upper panel in Fig. 4 shows that the binding of ^{125}I-VLDL is strongly inhibited by increasing amounts of VLDL and, at the highest concentration tested (2.7 μg of unlabelled competitor), the binding of the labelled species was only 51% ± 3 (S.E., $n = 3$) of the binding in the absence of competitor. However, in the presence of a similar quantity of LDL it was reduced to only 85 ± 3% of the control value. In contrast, when the effects of these two lipoprotein fractions were examined on the binding of ^{125}I-LDL (Fig. 4, lower panel) it

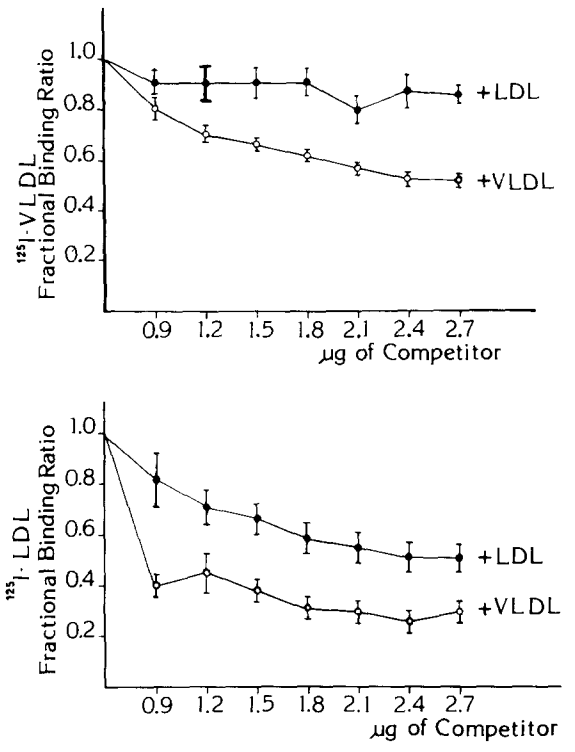

Fig. 4. Effect of increasing concentrations of either unlabelled VLDL or LDL on the binding of ^{125}I-VLDL (upper panel) or ^{125}I-LDL (lower panel) to human placental microvillous membranes. Values are plotted as mean ± S.E., $n = 3$ assays for VLDL and $n = 4$ for LDL. The amount of specific binding of the labelled lipoprotein measured in the presence of a competing lipoprotein is plotted as a fraction of the amount of specific binding measured under competitor-free conditions.

was seen that while substantial self-inhibition was observed, VLDL was even more effective in blocking the binding of ^{125}I-LDL, even at very low concentrations, causing an inhibition at the highest concentration tested to 31% ± 2 (S.E., $n = 4$) of the control binding value.

Discussion

Particular interest has been paid recently to the role of lipoproteins in steroidogenesis since it has been shown that cholesterol is the major source of steroidogenic substrate in the adrenal gland, ovary and possibly also in the testis [5]. The recent explosion in evidence for the cellular uptake of a range of large proteins by the mechanism of receptor-mediated endocytosis had its beginnings in the studies of Goldstein and Brown [6] on the binding and degradation of low-density lipoproteins by human fibroblasts. Their work gave rise to the concept of an LDL receptor which has now been characterized in a large number of different cell types [24]. Subsequently, the apoprotein moieties recognised by this receptor have been determined, and since they occur in several lipoprotein classes, the receptor is more correctly known as an apolipoprotein B,E receptor. However, it is not surprising that characterization of the placental lipoprotein receptor has to date mainly been attempted using LDL as the main ligand [7–9].

Recently, roles for HDL as an efflux acceptor for cholesterol destined for secretion by the liver and also as a means of delivering cholesterol to steroidogenic tissues for hormone synthesis have been described (see Ref. 25 for review). Studies with placental microvillous membranes have previously indicated only partial inhibition of LDL binding by HDL [9] probably due to small amounts of HDL_c which contains apolipoprotein E and which has a high affinity for the apolipoprotein B,E receptor [24,26]. Cummings et al. [8] using a mixed membrane preparation prepared from a placental homogenate, found a substantial binding of ^{125}I-HDL suggesting the presence of specific receptors. Winkel et al. [10] observed a linear uptake of ^{125}I-HDL up to a concentration of 1000 $\mu g \cdot dl^{-1}$, with negligible degradation, and concluded that the binding sites had a low affinity and high capacity. These sites could not, however, be ascribed to the microvillous membrane as these authors used a culture of mixed placental cells rather than pure trophoblast.

Another well-established role for the apolipoprotein B,E receptor in fibroblasts and macrophages, involves their ability to bind beta-VLDL found in hypertriglyceridaemic individuals, but not normal VLDL [27,28] via the apolipoprotein E moiety (see Ref. 29 for review). However, recent evidence in fibroblasts suggests that normal VLDL does in fact also bind to these receptors [12,24] as would be expected since normal VLDL contains both the apolipoproteins E and B.

The present study is the first demonstration of a high-capacity, high-affinity binding of normolipidaemic VLDL to the human placental microvil-

lous membrane. The amximal binding capacity was over 4-times greater than that observed for LDL and the affinity constant was one order of magnitude larger than that of LDL. It is assumed that apolipoprotein E moieties in the VLDL are responsible for these findings. It is of interest that the maximal binding capacity for LDL (270 ng per mg of membrane protein) is itself twice that reported by Alsat et al. [7] indicating that the additional membrane purification performed in our study was justified. The affinity constant reported here for LDL is similar to other published values for placental microvillous membranes [7,8] and also to that found for LDL in other cell types [24].

We also present here the first estimate of the affinity of HDL for the microvillous membrane receptor. The finding that it is about 100-times lower than that for VLDL is in agreement with its poor inhibitory effect on the uptake of LDL [7]. This implies that the saturable binding of HDL observed in a mixed placental membrane preparation [8] occurred at a site other than the microvillous membrane.

The similarity in the number of binding sites determined from the Scatchard plots (Figs. 1b, 2b and 3b), together with their linearity, suggests that there is a single population of sites for which each lipoprotein class is competing with differing affinities. This is further confirmed by the self- and cross-inhibition data (Figs. 4a and 4b) which clearly show that VLDL will preferentially bind to the receptors as it can displace ^{125}I-LDL more readily than LDL itself.

VLDL plays a major role in cholesterol transport to tissues and ultimately in cholesterol homeostasis [29]. The cholesterol ester content of VLDL is 11–14.2% of its mass and free cholesterol accounts for a further 5.1–8.4% [30]. Hence normal VLDL could be an important source of cholesterol for steroid synthesis. The total lipid content of VLDL increases almost four fold during gestation with the cholesterol content increasing from 9.4 mg · 100 ml^{-1} to 29.5 mg · 100 ml^{-1} [31], from the 28th week onwards [13]. Conversely, changes in LDL cholesterol fail to reach statistical significance [31]. Hence these changes, which result in the amount of cholesterol carried by VLDL approaching one third of that carried by LDL [31], together with the high affinity of the microvillous receptor for VLDL, strongly suggest that in contrast to other steroidogenic tissues, VLDL provides an important source of cholesterol to the placenta for progesterone synthesis, and could well supplement the function of the LDL pathway which is also for insertion of cholesterol into membranes.

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