Characterization of an atypical lipoprotein-binding protein in human aortic media membranes by ligand blotting

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By use of ligand-blotting techniques, this study investigated lipoprotein-binding proteins in human aortic smooth muscle. PAGE was performed under non-reducing conditions, and, using low-density lipoprotein (LDL) as ligand, with rabbit anti-apolipoprotein (apo) B and ^{125}I-labelled goat anti-rabbit IgG as primary and secondary antibodies respectively, we demonstrate that membranes from human aortic media (and cultured human smooth-muscle cells) contain a major lipoprotein-binding protein with an apparent molecular mass of 105 kDa. Anionized preparations (carbamoyl- and acetyl-) of LDL, which did not displace ^{125}I-LDL bound to the apo B,E receptor of cultured fibroblasts, were also recognized as ligands for the 105 kDa protein in aortic media membranes. LDL binding to 105 kDa protein was decreased in the presence of high density lipoprotein (HDL), although more than 100-fold molar excess of HDL was required to achieve 50% displacement of bound LDL. The LDL-binding activity of 105 kDa protein was inhibited by EDTA, and was also significantly decreased when samples were reduced by β-mercaptoethanol before electrophoresis. Monoclonal antibodies against apo B,E receptor reacted with partially purified bovine adrenal apo B,E receptor, but not with 105 kDa protein of human aortic media membranes. The spectrum of properties of this vascular smooth-muscle lipoprotein-binding protein binding are clearly distinct from those of other previously characterized lipoprotein-binding molecules.

INTRODUCTION

A number of receptors retain their ability to recognize their corresponding ligands after electrophoretic separation of proteins and blotting to nitrocellulose membrane [1]. Several lipoprotein receptor types, including the low-density lipoprotein (LDL) receptor [2], acetyl-LDL (scavenger) receptor [3], high-density lipoprotein (HDL) receptor [4–6] and LDL-receptor-related protein [7], have been detected by this ligand-blotting method. In each case the binding characteristics obtained in ligand-blotting studies closely resembled those determined in studies in intact cells, thus validating the use of ligand-blotting in the characterization of lipoprotein-binding proteins.

Several studies have recently demonstrated that lipoproteins are capable of eliciting intracellular signalling responses in a variety of cell types, including platelets, fibroblasts, endothelial cells and vascular smooth-muscle cells (VSMC) (reviewed in [8]). The responses elicited by lipoproteins are dose-dependent and saturable, indicating the probable, but not yet proven, involvement of specific receptors/binding sites in lipoprotein-stimulated signal transduction. Studies in cultured VSMC have also demonstrated that LDL-stimulated signalling is heparin-insensitive and involves a pertussis-toxin-sensitive guanine-nucleotide-binding protein (G-protein) [9], characteristics which would indicate that the signal-transduction-coupled LDL-recognition site is quite distinct from the 'classical' LDL receptor.

In order to investigate the existence of atypical lipoprotein-binding proteins in vascular smooth muscle, this study has applied the ligand-blotting method for characterization of lipoprotein-binding proteins in membranes isolated from de-endothelialized human medial aortic tissue. We demonstrate that these membranes contain a major lipoprotein-binding protein with a molecular mass of approx. 105 kDa, the properties of which differ markedly from those of the presently known types of lipoprotein receptors.

METHODS

Preparation of lipoproteins

LDL (density 1.019–1.063 g/ml) and HDL_3 (density 1.125–1.215 g/ml) were isolated from the plasma of healthy male humans by using sequential buoyant-density centrifugation techniques, with use of NaBr for density adjustments [9,10]. Apolipoprotein (apo) E-free HDL (HDL_3) was prepared from total HDL fraction (density 1.063–1.20 g/ml) by heparin–agarose affinity chromatography [11,12]. LDL preparations were free of protein fragments known to be generated during LDL oxidation [13]. LDL was acetylated (acetyl-LDL) as described by Basu et al. [14], and carbamoylated (carbamoyl-LDL) as described by Weisgraber et al. [15]. Complete chemical modification of LDL was validated by enhanced anodic electrophoretic mobility under non-denaturing conditions, as well as by the lack of ability to compete with native LDL for binding to the apo B,E receptor of fibroblasts (see the Results section, Figure 8). LDL was iodinated by the ICl method [16]. Protein concentrations were determined by the Lowry method, with BSA as standard.

Preparation of membranes from aortic tissue and cultured VSMC

Thoracic aortae were obtained *post mortem* (autopsies performed within 1–5 h of death) from three individuals (aged 5–65) who died from different accidental causes. Sarcolemmal membranes were isolated as described by Matlib [17] with minor modifications. Briefly, the vessels were split open and the lumen was scraped gently to remove adherent blood cells and the endothelial layer. The medial layer was then scraped away from the

Abbreviations used: LDL, low-density lipoprotein; HDL, high-density lipoprotein; apo, apolipoprotein; VSMC, vascular smooth-muscle cells; PMSF, phenylmethanesulphonyl fluoride; LRP, lipoprotein-receptor-related protein; LPL, lipoprotein lipase; PG, proteoglycan; GAG, glycosaminoglycan.

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adventitial layer, homogenized (Polytron) in ice-cold solution containing 250 mM sucrose, 0.05% (w/v) BSA, 1 mM phenylmethanesulphonyl fluoride (PMSF), 20 mM 3-(*N*-morpholino)-propanesulphonic acid (pH 7.4 at 4 °C), and centrifuged at 1000 *g* for 10 min at 4 °C. The supernatant was filtered through cheesecloth and centrifuged at 10000 *g* for 10 min at 4 °C. The resulting supernatant was centrifuged at 112000 *g* for 30 min at 4 °C, and the pellet was suspended in BSA-free homogenization solution. Confluent and quiescent cultures of human microarteriolar VSMC [18] were rinsed with PBS before being scraped into lysis buffer, containing 2 mM EDTA, 20 mM Tris/HCl (pH 7.4), 2 μg/ml leupeptin, 2 μg/ml aprotinin and 1 mM PMSF. After sonication, cell lysates were centrifuged at 100000 *g* for 30 min at 4 °C. The resulting pellets were suspended in 250 mM sucrose/1 mM PMSF/20 mM 3-(*N*-morpholino)-propanesulphonic acid (pH 7.4 at 4 °C). Medial and VSMC membrane preparations were divided into batches and stored in liquid N_2, and protein concentrations were determined by the Lowry method, with BSA as standard.

Preparation of partially purified apo B,E receptors

Solubilization and partial chromatographic purification of apo B,E receptor from human liver and bovine adrenal cortex was performed as described [19]. These preparations were generously given by Dr. V. Tsibulsky, Cardiology Research Center, Moscow, Russia.

Culture of fibroblasts and competition binding

Conditions for ^{125}I-LDL binding to confluent and quiescent cultures of human skin fibroblasts [20] have been detailed previously [9]. Briefly, the binding of ^{125}I-LDL (at 5 μg/ml), without or with inclusion of unlabelled native LDL, acetyl-LDL or carbamoyl-LDL, was carried out for 4 h at 4 °C in Dulbecco's modified Eagle's medium containing 0.1% BSA. Non-specific binding of ^{125}I-LDL was determined by including excess (1 mg/ml) unlabelled LDL in parallel series of dishes. After washing, monolayers were incubated in the presence of heparin (10 mg/ml), and the displaced radioactivity was taken as the measure of binding to apo B,E receptor [16].

SDS/PAGE, ligand blotting and immunoblotting

Unless otherwise specified (Figure 4), PAGE was performed under non-reducing conditions. Portions of membrane preparations were solubilized in sample buffer without β-mercaptoethanol and heated for 5 min at 85 °C. Proteins (100 μg loaded per slot) were electrophoresed on SDS/polyacrylamide (8%) gels [21]. A molecular-mass standard mixture (SDS-6H: Sigma Chemical Co., St. Louis, MO, U.S.A.), consisting of carbonic anhydrase (29 kDa), egg albumin (45 kDa), phosphorylase *b* (97 kDa), β-galactosidase (116 kDa) and myosin (205 kDa) was used for calibration. After electrophoretic transfer of proteins to nitrocellulose membranes [22] and detection of protein by Ponceau S staining, blots were destained in Tris-buffered saline (TBS; 150 mM NaCl, 50 mM Tris/HCl, pH 7.4). Unless otherwise specified (Figure 3), TBS containing 5% (w/v) delipidated milk (TBSM) was used in all pre-blocking, incubation and washing procedures. The milk (Rapilait, Zurich, Switzerland) contained 34 g of milk protein, 0.5 g of milk fat, 53 g of milk sugar and 8 g of mineral substances per 100 g of dry powder. For ligand blotting, blots were preincubated for 1 h in TBSM, and then incubated for 2 h with the required concentrations of LDL in TBSM. After repeated washes (5 × 5 min) with TBSM, blots were incubated for 2 h with rabbit anti-apo B antiserum (Calbiochem; dilution 1:200 in TBSM), washed again and then incubated for 2 h with ^{125}I-labelled goat anti-rabbit IgG. For immunoblotting, blots were preincubated for 1 h in TBSM, incubated for 2 h with either 1 μg/ml IgG C7 or 1 μg/ml IgG F13 (both monoclonal antibodies against the apo B,E receptor), washed again and then incubated for 2 h with ^{125}I-labelled goat anti-mouse IgG as second antibody. IgG C7, which recognizes the ligand-binding domain of the apo B,E receptor [23] was obtained commercially (Calbiochem, Laufelfingen, Switzerland), and IgG F13 was generously given by Dr. V. Tsibulsky, Cardiology Research Center, Moscow, Russia. Radiolabelling of second antibodies (specific radioactivities 10000–15000 c.p.m./ng) was performed by the Iodogen method [24]. After incubation with second antibodies, blots were repeatedly washed with TSBM, dried and exposed to X-ray film. Immunoreactive proteins (immunoblots) and lipoprotein-binding proteins (ligand blots) were made visible on autoradiographs. After localization of protein-bound LDL on ligand blots, the 105 kDa radiolabelled band was excised and counted for radioactivity in a γ-radiation counter. To control for residual non-specific binding of LDL to nitrocellulose, two background sections of nitrocellulose of the same size as the lipoprotein-binding band were also excised from each strip and counted for radioactivity. The mean value of this background radioactivity was subtracted from that radioactivity associated with the 105 kDa band, and resulting values were taken to represent specific binding. All experiments presented herein have been performed by using an alternative method of detection, namely the use of anti-rabbit IgG peroxidase-conjugated secondary antibodies (Calbiochem) and staining with diaminobenzidine as substrate with metal ion enhancement. The results obtained thereby (not shown) were qualitatively identical.

RESULTS

PAGE of medial tissue membrane proteins was performed under non-reducing conditions in the presence of SDS, and lipoprotein-binding proteins were detected by sequential incubation of Western blots with LDL, primary anti-apo B antibodies and secondary ^{125}I-labelled anti-IgG antibodies. Autoradiographs of blots revealed selective binding of LDL to a protein with an apparent molecular mass of approx. 105 kDa (Figure 1). The amount of radioactivity associated with the 105 kDa-protein was sufficient for quantification of binding (as in Figures 3–7) by excision and γ-radiation counting. This 105 kDa band was not detectable on parallel Coomassie-stained polyacrylamide gels (results not shown). The appearance of the 105 kDa band on autoradiograms was typically broad and diffuse.

Although other minor radiolabelled bands were also evident after prolonged exposure to X-ray film (e.g. see Figures 4, 5 and 6), only proteins of ~ 130 kDa and ~ 90 kDa consistently exhibited minor LDL-binding activity. LDL-binding activity of these proteins was too minimal to permit further characterization, although ligand-blotting studies on partially purified adrenal LDL receptors have identified the 130 kDa and 90 kDa LDL-binding proteins as the mature apo B,E receptor and its precursor, respectively [2]. The minor binding activity of these proteins correlates well with known low levels of expression of LDL receptor in human cells *in vivo* [25]. The three different aortic membrane preparations used in this study yielded consistent results with respect to major LDL-binding activity of the 105 kDa protein and very minor LDL-binding activity of the 130 kDa protein. In membranes isolated from quiescent cultured human microarteriolar (Figure 1, inset) or rat aortic VSMC (results not shown), major LDL-binding activity was also localized to a 105 kDa protein. The apparent absence of lipoprotein binding to

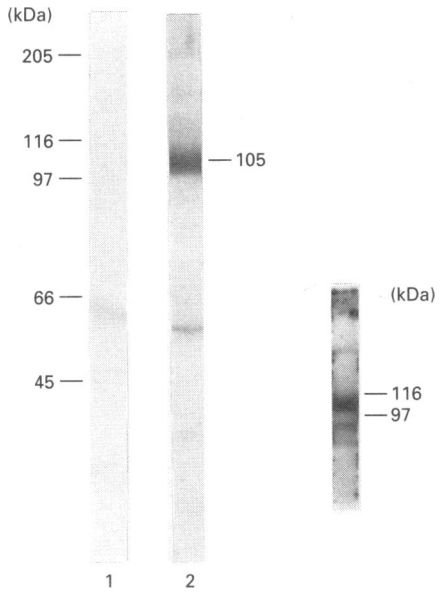

Figure 1 Ligand-blot detection of a 105 kDa LDL-binding protein in human aortic media

Membrane proteins from human aortic media were electrophoresed under non-reducing conditions in SDS/8%-polyacrylamide gels, and transferred to nitrocellulose. After blocking of non-specific binding, nitrocellulose strips were incubated for 2 h at room temperature either without (lane 1) or with (lane 2) 25 μg/ml LDL. LDL-binding protein was detected by incubation with rabbit anti-apo B and ^{125}I-labelled goat anti-rabbit IgG, followed by autoradiography, as described in the Methods section. A typical autoradiogram is shown; the relative electrophoretic mobilities of the molecular-mass standards (kDa) are indicated on the left. The autoradiogram inset illustrates specific binding of LDL to 105 kDa protein in membranes from cultured human smooth-muscle-cell membranes; the relative mobilities of 97 kDa and 116 kDa molecular-mass standards are also indicated.

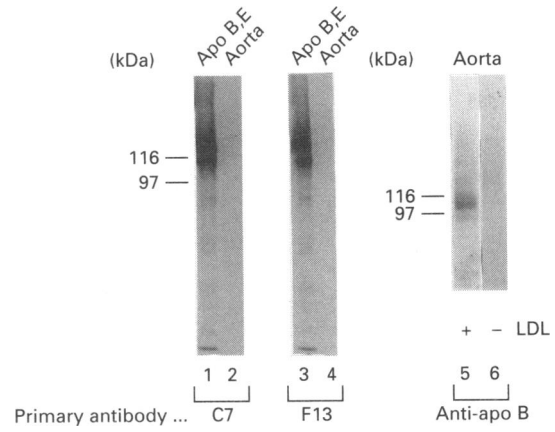

Figure 2 Antibodies to apo B,E receptor do not recognize 105 kDa protein

Human aortic media membranes (lanes 2, 4, 5 and 6) and partially purified bovine adrenal apo B,E receptor (lanes 1 and 3) were co-electrophoresed and blotted on to nitrocellulose. Blots were analysed either for immunoreactivity to anti-apo B,E receptor antibodies, C7 (lanes 1 and 2) and F13 (lanes 3 and 4), or for LDL binding (lanes 5 and 6). For the latter, strips were incubated with (+, lane 5) and without (−, lane 6) 25 μg/ml LDL before detection of LDL-binding protein with anti-apo B as the primary antibody. The relative electrophoretic mobilities of 97 kDa and 116 kDa molecular-mass standards are indicated. Experimental details are given in the Methods section.

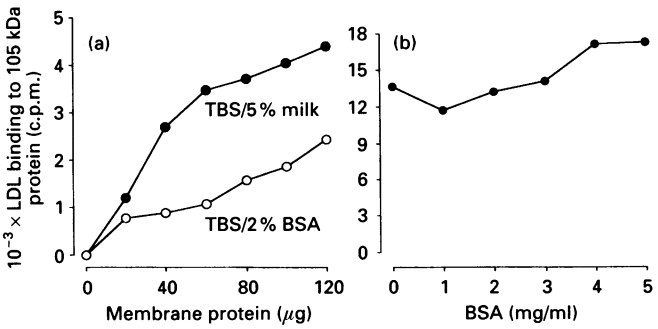

Figure 3 Dependence of LDL binding to the 105 kDa protein on the type of blocking solution

(**a**) Different amounts of aortic-media membrane protein were separated on SDS/polyacrylamide gels and blotted on to nitrocellulose. Non-specific binding was blocked with TBS buffer containing either 2% BSA (○) or 5% milk (●), and blots were incubated for 2 h in the presence of 25 μg/ml LDL. (**b**) In a separate experiment, a constant amount of aortic membrane protein (40 μg) was loaded into wells, non-specific binding was blocked with TBS containing 5% milk, and incubations of blots with LDL included the indicated concentrations of BSA. Finally, blots were washed. Data for specific binding of LDL to 105 kDa protein are presented. Experimental and analytical details are given in the Methods section.

130 kDa protein in quiescent VSMC cultures is consistent with their non-proliferative state and low cholesterol requirements [25].

The electrophoretic mobility of LDL-binding protein in aortic membranes (105 kDa) was quite different from that (LDL binding localized to 130 kDa protein) of purified human hepatic or bovine adrenal LDL receptor (results from ligand blots not shown). To distinguish further between 130 kDa (apo B,E receptor) and 105 kDa proteins, aortic membranes and partially purified bovine adrenal LDL receptors were compared for immunoreactivity with two monoclonal anti-apo B,E receptor antibodies (IgG C7 and IgG F13). Although both C7 and F13 antibodies recognized bovine adrenal apo B,E receptor, they did not interact with the 105 kDa LDL-binding protein in aortic membranes (Figure 2). In accordance with the apparent absence of LDL binding to 130 kDa protein on ligand blots, there was negligible immunoreactivity of 130 kDa protein in aortic membranes to C7 or F13 antibodies (Figure 2).

In previous ligand-blotting studies, solutions of BSA [2,3,7,26] or delipidated milk [5,6] have been used to block non-specific lipoprotein binding to nitrocellulose. We compared the effect of the two types of blocking solutions on the binding of LDL to the 105 kDa protein. For both albumin and milk solutions the amount of LDL specifically bound to the 105 kDa protein increased with increasing amounts of membrane protein loaded on to gels, but the absolute values were significantly higher in the presence of milk (Figure 3a). This may be due to a more complete removal of SDS, or to a more correct renaturation of the lipoprotein-binding protein in the presence of milk. Since inclusion of BSA (1–5 mg/ml, in milk-containing incubation solution) did not decrease LDL binding to 105 kDa protein (Figure 3b), differential effects of BSA and milk are unlikely to reflect a relative inhibitory effect of BSA on lipoprotein binding. In all subsequent experiments delipidated milk was used to block non-specific binding.

The specific binding of LDL to 105 kDa protein was concentration-dependent (Figure 4a), and the 105 kDa band could be detected at concentrations of LDL as low as 1 μg/ml (Figure 4c). The concentration-dependent profile for LDL binding to 105 kDa protein in aortic membranes is comparable with that previously found for ^{125}I-LDL binding to cultured VSMC [9].

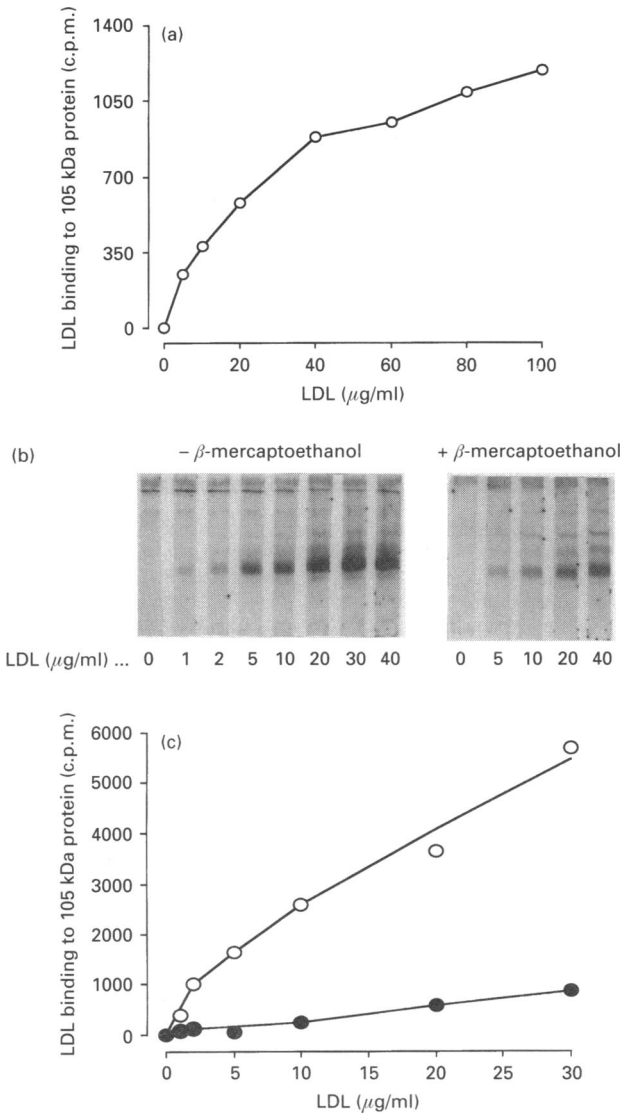

Figure 4 Concentration-dependence of LDL binding of the 105 kDa protein, and the effect of thiol-group reduction of LDL binding

Aortic membrane proteins were solubilized in sample buffer either without (○; **a**, **c**) or with (●; **c**) inclusion of β-mercaptoethanol. Blots were incubated with the indicated concentrations of LDL (**a**: 5–100 μg/ml; **b** and **c**: 1–30 μg/ml) for 2 h at room temperature. LDL binding was revealed by autoradiography (**b**) and specific LDL binding to 105 kDa protein was quantified (**a** and **c**). Experimental and analytical details are given in the Methods section. The data in each panel are from three independent experiments. Two other experiments using peroxidase-conjugated secondary antibodies yielded results qualitatively comparable with those presented.

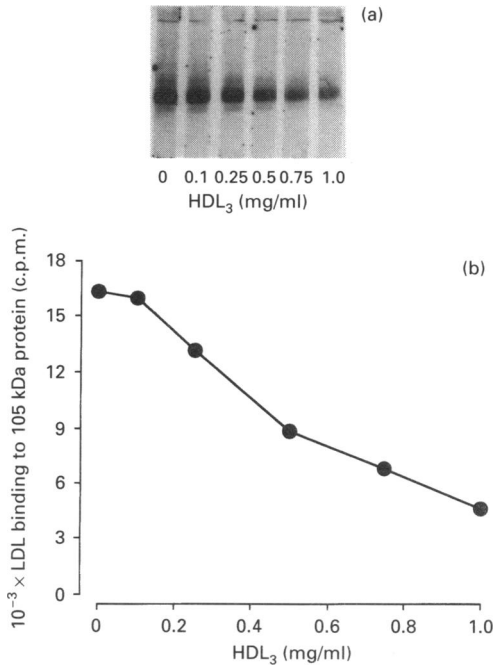

Figure 5 Influence of HDL_3 on binding of LDL to the 105 kDa protein in aortic membranes

Blots were incubated for 2 h in the presence of 25 μg/ml LDL, together with the indicated concentrations of HDL_3. LDL binding was revealed by autoradiography (**a**) and specific LDL binding to 105 kDa protein was quantified (**b**). Experimental and analytical details are described in the Methods section. The data are representative of two experiments using ^{125}I-labelled secondary antibodies. Qualitatively similar results were obtained in three other experiments which used peroxidase-conjugated secondary antibodies for detection of LDL binding.

The estimated affinity for LDL binding to 105 kDa protein (~ 40 μg/ml) is also similar to the calculated K_d (47.3 ± 15.1 μg/ml) of the low-affinity ^{125}I-LDL-binding site in VSMC [9]. The affinity for binding to 105 kDa protein is markedly lower than that (~ 1 μg/ml) determined for 'classical' LDL receptor on ligand blots [2]. Treatment of membrane proteins with the thiol reducing agent β-mercaptoethanol before electrophoresis significantly decreased the LDL-binding activity of the 105 kDa protein (Figures 4b and 4c).

In order to assess lipoprotein specificity of the 105 kDa protein, blots were incubated in the presence of LDL together with increasing concentrations of HDL. To avoid any ambiguity which might arise from the presence of HDL particles containing apo E, a known ligand of apo B,E receptor, we used a subclass of HDL (HDL_3, density 1.125–1.215 g/ml) that is known to be essentially free of this apoprotein [11]. HDL_3 was capable of decreasing LDL binding to 105 kDa protein in a concentration-dependent manner, although a large excess of HDL_3 was required for such displacement (Figure 5). More than a 100-fold molar (or 20-fold protein) excess of HDL_3 (~ 600 μg/ml) was required to achieve 50% inhibition of the binding of LDL (at 25 μg/ml). These data demonstrate a significant selectivity of the 105 kDa lipoprotein-binding protein for LDL. Binding of LDL to the 'classical' LDL (apo B,E) receptor is dependent on the presence of bivalent cations and can be inhibited by EDTA [2,25]. The data in Figure 6 indicate that EDTA also inhibits LDL binding to the 105 kDa protein. High concentrations of $CaCl_2$ slightly diminished LDL-binding activity of this protein (Figure 7).

Selective chemical modification (e.g. acetylation, carbamoylation) of lysine residues in LDL abolishes binding to apo B,E receptor [27]. This has been demonstrated by both ligand-blotting studies on membrane preparations [2] and binding studies in cell cultures [14,16,28]. The inability of anionized LDL to bind to apo B,E receptor is illustrated in the competition-binding experiment presented in Figure 8(a), whereby in cultured quiescent fibroblasts native LDL fully competed for bound ^{125}I-LDL (heparin-releasable), whereas displacement of ^{125}I-LDL by either acetyl-LDL or carbamoyl-LDL was negligible. However, in ligand-blot experiments on aortic medial membranes we found that, like native LDL, both acetyl-LDL and carbamoyl-LDL

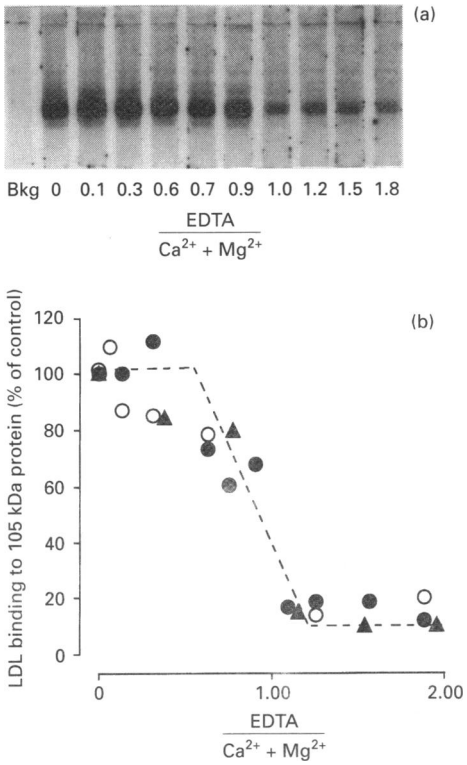

Figure 6 Effect of EDTA on LDL-binding activity of the 105 kDa protein in aortic membranes

Blots were incubated for 2 h in either 5% milk/TBS alone (Bkg) or in 5% milk/TBS containing 25 μg/ml LDL and without or with inclusion of different concentrations (2–30 mM) of EDTA. The 5% milk/TBS incubation solution was analysed for ionic composition with a biochemical analyser (Spectrum; Abbot Laboratories, Abbot Park, IL, U.S.A.) by using reagent kits from the same company. Concentrations of K^+ and Na^+ were determined by using ion-selective electrodes, and concentrations of Ca^{2+} (14 mM) and Mg^{2+} (2.15 mM) were determined colorimetrically by using *o*-cresolphthalein and calmagite respectively). The abscissa values in (**a**) and (**b**) indicate the ratio of the concentration of EDTA to the concentration of bivalent cations (Ca^{2+} plus Mg^{2+}). A typical autoradiograph is presented in (**a**). Specific binding of LDL to 105 kDa protein was quantified (**b**). The different symbols in (**b**) (○, ● and △) represent data from there independent experiments. Experimental and analytical details are described in the Methods section. Three other experiments using peroxidase-conjugated secondary antibodies yielded qualitatively similar results.

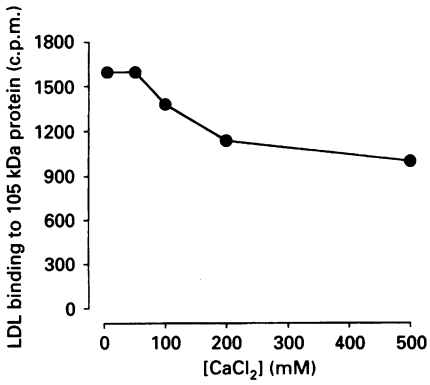

Figure 7 Effect of high concentrations of $CaCl_2$ on LDL-binding activity of the 105 kDa protein in aortic membranes

Blots were incubated for 2 h in the presence of 25 μg/ml LDL and increasing concentrations of $CaCl_2$. LDL binding was revealed by autoradiography and LDL specifically bound to 105 kDa protein was quantified. Experimental details are given in the Methods section.

bound specifically and selectively to 105 kDa protein (Figure 8b). Anionized LDL, but not native LDL, are recognized ligands for 'scavenger receptor' (220–260 kDa) [27]. However, binding of either acetyl-LDL or carbamoyl-LDL to a protein of 220–260 kDa was not detected in human aortic medial membranes.

DISCUSSION

This study has employed a previously described method for quantitative detection of protein-bound LDL on Western blots by using ^{125}I-labelled antibodies [2,3]. Using LDL as the lipoprotein ligand, we have demonstrated that human aortic membranes contain a major lipoprotein-binding protein with an apparent molecular mass of 105 kDa. Several lines of evidence support that the lipoprotein-binding property of 105 kDa protein is mediated by specific recognition processes rather than via a non-specific interaction of lipid particle with some hydrophobic cellular protein. Firstly, LDL binding was not inhibited by either BSA or the complex mixture of proteins present in delipidated milk. Secondly, the lipoprotein-binding activity was highly specific for LDL as opposed to HDL_3, a 100-fold molar excess of HDL_3 being required to produce a 50% loss of LDL binding. Thirdly, as shown for LDL binding to apo B,E receptor [2] and acetyl-LDL (scavenger) receptor [3], LDL-binding activity of 105 kDa protein was markedly diminished after reduction of disulphide bonds. In the case of the apo B,E receptor, intact cysteine residues are necessary for proper folding of protein domains containing clusters of negatively charged residues [29]. By analogy, therefore, recognition of LDL by 105 kDa protein may also require a certain tertiary structure of the protein, rather than some primary or secondary amino acid sequence such as the lipid-binding amphipathic helixes which are thought to mediate association of exchangeable serum apolipoproteins with lipids [30]. Fourthly as demonstrated for the specific binding of LDL to apo B,E receptor [26,31,32] and lipoprotein-receptor-related protein (LRP) [7], LDL-binding activity of 105 kDa protein was Ca^{2+}-dependent. Finally, high concentrations of Ca^{2+} (0.5 M) diminished LDL-binding activity of 105 kDa protein, indicating an involvement of ionic rather than hydrophobic interactions, the latter being favoured under conditions of high ionic strength.

The identity of the 105 kDa protein is not clear, and both similarities and differences exist with respect to the characteristics of LDL binding to 105 kDa protein and those properties of previously characterized LDL receptors. LRP has been reported to bind certain lipoprotein species in ligand-blotting studies, but its molecular mass (under electrophoretic conditions similar to those used in this study) was determined to be ~ 515–600 kDa [7]. Another lipoprotein receptor capable of lipoprotein binding on ligand blots is the scavenger receptor [3]. However, this receptor has an electrophoretic mobility corresponding to a molecular mass of ~ 260 kDa [3], and does not recognize native LDL [27]. Furthermore, fucoidin, a specific blocker of the scavenger receptor [27], did not inhibit LDL binding to 105 kDa protein (results not shown). Use of scavenger receptor ligands, namely acetyl-LDL and carbamoyl-LDL, in our ligand-blotting experiments did not reveal detectable levels of this protein in human aortic membranes. The apparent absence of scavenger receptor in medial membranes is consistent with previous observations [33] of a negligible constitutive expression of scavenger receptor by smooth-muscle cells. In cultures of either smooth-muscle cells or fibroblasts, detection (ligand- or immuno-blotting) of scavenger receptor activity required pretreatment of cultures with phorbol ester [33]. Such data permit exclusion of the participation of either LRP or scavenger receptor in the human aortic membrane lipoprotein-binding activity described herein.

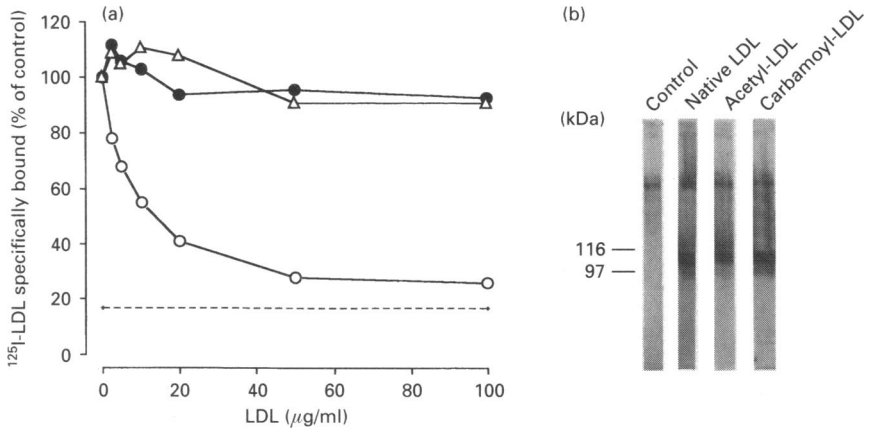

Figure 8 The 105 kDa protein binds native and anionized LDL preparations

(**a**) Human fibroblasts were incubated with 5 μg/ml ^{125}I-LDL and without or with inclusion of the indicated concentrations of unlabelled native LDL (○), acetyl-LDL (●) or carbamoyl-LDL (△). Heparin-releasable ^{125}I-LDL binding was measured, and data for binding in the presence of unlabelled lipoproteins are expressed relative (percentage) to ^{125}I-LDL bound in the absence of any competitor (100%; absolute value of 8.7 ng/mg of cell protein). The broken line represents non-specific binding. (**b**) Nitrocellulose blots of human aortic media membranes were incubated for 2 h either in the absence of lipoprotein (Control) or in the presence of native LDL, acetyl-LDL or carbamyl-LDL (each at 25 μg/ml). After incubation with primary anti-apo B and secondary ^{125}I-labelled antibodies, LDL binding was revealed by autoradiography. The relative electrophoretic mobilities of 97 kDa and 116 kDa molecular-mass standards are indicated. All experimental details are given in the Methods section.

LDL binding to both the 105 kDa protein and 'classical' apo B,E receptor has two common features, namely a requirement for intact disulphide bonds as well as the presence of bivalent cations. We suppose that the two proteins are not identical, since they differ with respect to both affinity for LDL binding (the apparent affinity of 105 kDa protein for LDL is lower than that established for apo B,E receptor) and molecular mass. Under non-reducing electrophoretic conditions, the apparent molecular masses of mature apo B,E receptor and its precursor are 130 kDa and 90 kDa respectively [2]. Differences in apparent molecular mass cannot be explained by some artefact of our electrophoretic procedures, since in direct comparison (after co-electrophoresis) of aortic membrane protein and partially purified hepatic or adrenal apo B,E receptors lipoprotein-binding activities were located at discrete distinct bands. We are unaware of any data indicating that apo B,E receptor may be fragmented without a concomitant loss of binding activity, and the 105 kDa protein is thus unlikely to be a proteolytic fragment of apo B,E receptor, artificially generated in the course of membrane isolation. Another difference between apo B,E receptor and 105 kDa protein relates to lipoprotein selectivity. Competition-binding studies in intact fibroblasts [34] and membranes from fibroblasts [31] have shown that 50% displacement of LDL by HDL requires more than a 400-fold molar excess of HDL. Our data demonstrate that a 100-fold molar excess of essentially apo E-free HDL_3 ($d = 1.125$–1.215) was sufficient to produce 50% inhibition of LDL-binding activity of the 105 kDa protein in aortic membranes, suggesting a less stringent lipoprotein-binding specificity. The HDL ($d = 1.085$–1.215) used in the studies on fibroblasts includes apo E-containing HDL particles, and one can therefore expect that apo E-free HDL would have been even less efficacious in displacing LDL. In a later study on purified apo B,E receptors [32], bound LDL was minimally displaced (~4%) by a 400-fold molar excess of essentially apo E-free HDL_3. We have also demonstrated that 105 kDa protein can bind not only native LDL but also chemically modified preparations of LDL (acetyl-LDL and carbamoyl-LDL) that are not recognized by the apo B,E receptor. Furthermore, monoclonal antibodies against apo B,E receptor (IgG C7 or IgG F13) interacted with partially purified apo B,E receptor, but not with 105 kDa protein of aortic membranes. Taken together, these data strongly suggest that the 105 kDa protein is distinct from the 'classical' apo B,E-recognizing receptor.

Another type of lipoprotein receptor which can specifically recognize HDL has been found previously in several cell types, including VSMC [12]. It was demonstrated that this receptor selectively binds HDL and that expression of specific HDL binding can be enhanced by pretreatment of cells with cholesterol [35]. An HDL-binding protein, with an apparent molecular mass of 105–110 kDa and which can be up-regulated by cholesterol, has been detected by ligand blotting [6]. Clearly, the size of this HDL receptor is very close to that of the lipoprotein-binding protein detected in human aortic media membranes. However, several characteristics of the lipoprotein-binding activity of 105 kDa protein, including a significant selectivity for LDL (100-fold molar excess of HDL_3 produces only 2-fold decrease in LDL binding), a prominent loss of binding activity after reduction of disulphide bonds, and a dependence on bivalent cations, contrast with the previously established properties of the HDL receptor [4–6,12,35,36].

Studies in hepatoma cells have revealed the existence of another lipoprotein-binding protein, identified as nucleolin or a nucleolin-like protein which is partially expressed on the cell surface [37]. The similarities of nucleolin to the 105 kDa lipoprotein-binding protein in aortic membranes include its molecular mass (~109 kDa), a greater selectivity for LDL (compared with HDL) and a lower affinity (compared with apo B,E receptor) for LDL. However, in contrast with LDL-binding activity of 105 kDa protein, the LDL-binding activity of nucleolin is insensitive to both EDTA and thiol-group-reducing agents [37].

Another lipoprotein-binding protein of interest is lipoprotein lipase (LPL), an essential enzyme of lipid metabolism and a normal constituent of the arterial wall. LPL has been shown to enhance binding of lipoproteins to cells and the extracellular matrix, probably by mediating bridge formation between lipoprotein particles and cell-surface/extracellular-matrix-contained

heparan sulphate [38–40]. Although LPL is primarily located on the luminal endothelial cell surface of the vessel wall [41], it can also be transported across the endothelial cell layer via both non-specific fluid-phase transcytosis and a specific saturable transport system [42]. Additionally, aortic smooth-muscle cells have been shown to express LPL mRNA [43]. We can, however, confidently exclude the participation of LPL in the major lipoprotein-binding activity of our de-endothelialized aortic membrane preparations, since not only does LPL have a markedly lower molecular mass (50 kDa) [44], but LPL-mediated LDL binding is almost completely independent of Ca^{2+} [40].

Arterial-wall sulphated proteoglycans (PGs), complex macromolecules in which sulphated glycosaminoglycan (GAG) chains are linked to a protein core, are also capable of binding lipoproteins [45]. The binding of LDL to sulphated PGs is believed to occur via ionic interactions between the negatively charged sulphate groups in the saccharide units of GAGs and the positively charged lysine and arginine residues of apo B-100 [46,47]. LDL is able to interact with several arterial-wall PGs, including the dermatan sulphate, heparan sulphate and chondroitin sulphate types, and even within the same PG/GAG family there is considerable heterogeneity with respect to size, charge and polysaccharide content [46,48]. Since we consistently detected only one major lipoprotein-binding band on ligand blots, a participation of PG/GAG interactions in the lipoprotein-binding activity of 105 kDa protein appears most unlikely. Additionally, binding of LDL to PGs/GAGs is strongly dependent on ionic strength, with complete inhibition at concentrations of NaCl greater than ~ 0.15 M [47,49]. This feature contrasts starkly with the minimal effect of 0.5 M $CaCl_2$ on 105 kDa-protein LDL-binding activity.

Some of the features described here, of lipoprotein binding to the 105 kDa protein in aortic membranes, are generally regarded as characteristic of specific lipoprotein receptors. These include lipoprotein selectivity, a dependence on bivalent cations and the requirement for a certain tertiary protein structure. Nevertheless, in a comparative appraisal of the present and previously published data, it is evident that the spectrum of properties of the 105 kDa lipoprotein-binding protein is distinct from those of previously identified lipoprotein-binding molecules. At present we cannot conclude that the 105 kDa protein is a completely novel lipoprotein-binding molecule, and must still consider that it could represent a modification of some known lipoprotein receptor.

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