Very low density lipoprotein receptor binds apolipoprotein E2/2 as well as apolipoprotein E3/3

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Received 8 March 1996; revised version received 15 April 1996

Abstract The VLDL receptor, a newly identified lipoprotein receptor, recognizes apoE containing lipoproteins. The human VLDL receptor was overexpressed in ldlA-7, a mutant Chinese hamster ovary cells lacking LDL receptors. Each VLDL obtained from a normolipidemic subject with two ε3 or ε2 alleles similarly competed for the binding of radiolabeled rabbit β-VLDL to the VLDL receptors. The anti-apoE monoclonal antibody 1D7, which inhibited binding of apoE3 to the LDL receptors, failed to compete for the binding of VLDL (apoE3 or apoE2) to the VLDL receptors. Results indicate that the binding site of apoE on the VLDL receptor may differ from its binding site on the LDL receptor.

Key words: Lipoprotein; Lipoprotein receptor; Very low density lipoprotein; VLDL receptor; Apolipoprotein E

1. Introduction

The VLDL receptor has been identified by the cloning of a cDNA from a rabbit heart cDNA library [1]. The amino acid sequence of the VLDL receptor has a striking homology to the low density lipoprotein (LDL) receptor. The only marked structural difference between them is the number of cysteine-rich repeats in the ligand binding domain. The VLDL receptor has eight repeats whereas the LDL receptor has seven. The VLDL receptor binds with high affinity to particles containing apolipoprotein E (apoE), but not LDL [1,2]. The human VLDL receptor has been isolated from monocytic leukemia cells (THP-1) and characterized; its expression is not down-regulated by sterols [3].

Type III hyperlipoproteinemia is a genetic lipid disorder that is characterized by the accumulation of cholesterol-rich chylomicron remnants and hepatic β-VLDL. The affected patients exhibit xanthomas, an increased propensity for premature coronary and peripheral vascular disease, or both. Most patients with type III hyperlipoproteinemia are homozygous for a mutant form of apoE, apoE2, that differs from normal apoE (apoE3) by a single amino acid substitution (Cys^{158} to Arg) [4]. β-VLDL is rich in apoE2, which is dysfunctional with respect to binding to hepatic receptors leading to the accumulation of β-VLDL in plasma. If a β-VLDL contributes to atherosclerosis, the formation of foam cells must occur by a mechanism other than one that involves the LDL receptor. In the present study, we examined whether the VLDL from a subject homozygous for apoE2 binds the human VLDL receptor as well as the VLDL from a subject homozygous for apoE3.

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Abbreviations: VLDL, very low density lipoprotein; LDL, low density lipoprotein; apo, apolipoprotein; β-VLDL, β-migrating very low density lipoprotein; LPL, lipoprotein lipase; PCR, polymerase chain reaction; LPDS, lipoprotein-deficient serum; LRP, LDL receptor-related protein; SDS, sodium dodecyl sulfate

2. Materials and methods

2.1. Materials

Monoclonal antibodies directed against apoE were generously provided by Dr. Yves Marcel (Ottawa Heart Institute, Canada). Two anti-apoE antibodies were used: 1D7, an antibody that especially inhibits binding by apoE to the LDL receptor, and 6C5, an antibody that reacts with an epitope that is not involved with receptor recognition [5]. Antibodies were used after they were affinity purified from ascitic fluid. 1D7 was confirmed to inhibit binding by [^{125}I]recombinant apoE3 to human LDL receptor-overexpressing cells.

2.2. Lipoprotein preparation

VLDL (d<1.006 g/ml) was isolated by ultracentrifugation from the plasma of a normolipidemic Japanese subject homozygous for the two ε2 (Cys^{158} to Arg) alleles (N.Y.) or a subject with two ε3 alleles (K.M.) [6]. ApoE genotypes were determined by restriction isotyping [7]. The concentration of apoE in human VLDL was determined by the a single radial immunodiffusion method (APO E PLATE, Daiichi, Tokyo, Japan). The subject homozygous for ε2 alleles was normolipidemic but her plasma exhibited a broad β pattern by polyacrylamide gel electrophoresis. Rabbit β-VLDL (d<1.006 g/ml) was isolated from the plasma of rabbits fed a diet containing 0.5% cholesterol. Bovine lipoprotein-deficient serum (LPDS) (d>1.215 g/ml) was isolated from bovine fetal serum. Lipoproteins were labeled with ^{125}I by the method of Bolton and Hunter [8]. The final specific activity was 200–780 cpm/ng protein and more than 96% of the ^{125}I radioactivity was precipitable by incubation with 10% (w/v) trichloroacetic acid.

2.3. Isolation of transfected cells

Plasmids encoding the human VLDL receptor (ph-VLR2) or pSV2-neo, which contains the neomycin resistance gene, were transfected to ldlA-7 cells (mutant Chinese hamster ovary cells that lack LDL receptors) which was kindly provided by Dr. Monty Krieger (Department of Biology, Massachusetts Institute of Technology, Cambridge, MA) using lipofectin as described previously [1-3,9,12]. Positive cell clones for the human VLDL receptor were designated VLDL-R cells. A transfectant with only pSV2-neo was used for control cells (Neo).

2.4. Cell culture and ligand binding assays

For experiments designed to assay the ligand binding activity of the transfectants, ^{125}I-labeled lipoproteins were added to the cells in Ham's F-12 medium supplemented with 5% LPDS. After 4 h at 37°C, the cell association (binding and internalization) of [^{125}I]lipoproteins was measured as described by Goldstein and Brown [10]. Cellular protein was measured according to the method of Lowry et al. [11]. To exclude the effect of LRP (LDL receptor-related protein), specific activity was determined by subtracting the radioactivity associated with Neo cells from that associated with VLDL-R cells [12]. All binding assays were performed in triplicate.

PII S0014-5793(96)00439-5

2.5. Solubilization of cell membrane and ligand blotting assay

Membrane fractions of transfected cells were prepared by the method of Hofmann et al. [13]. Ligand blotting was performed with ^{125}I-labeled lipoproteins as described before [12].

3. Results

3.1. Binding of VLDL of apoE3/3 and apoE2/2 to the VLDL receptor

Human fasted [^{125}I]VLDL from normolipidemic subjects has been shown to bind the VLDL receptor with lower affinity compared with β-VLDL from cholesterol-fed rabbits [12]. Competitive binding assays in cell culture were performed using [^{125}I]rabbit β-VLDL instead of human VLDL. The VLDL of apoE3/3 and the VLDL of apoE2/2 competed for the binding of radiolabeled rabbit β-VLDL to the VLDL receptor with similar affinities (Fig. 1). Repeated experiments (n=3) could not distinguish the ability of the two kinds of VLDL to compete with the binding of [^{125}I]β-VLDL.

A ligand blot assay was performed to ensure whether the VLDL binds to the VLDL receptor (Fig. 2). Ligand blot was employed because this method has higher sensitivity and specificity than the cell culture binding assay. Both the VLDL of apoE3/3 and the VLDL of apoE2/2 certainly bound to the VLDL receptor. Additional bands such as LRP were not visible. Although the intensity of the band was greater in the apoE2/2 lane in this experiment, this was not always shown in the repeated experiments (Fig. 3).

3.2. Effect of anti-apoE antibodies

The effect of 1D7, an antibody that especially inhibits binding by apoE to the LDL receptor, and 6C5, an antibody that reacts with an epitope that is not involved with receptor recognition, on the binding of [^{125}I]VLDL of apoE3/3 to the VLDL receptor was examined by ligand blot assay and cell culture binding assay. In the case of ligand blot assay (Fig. 3),

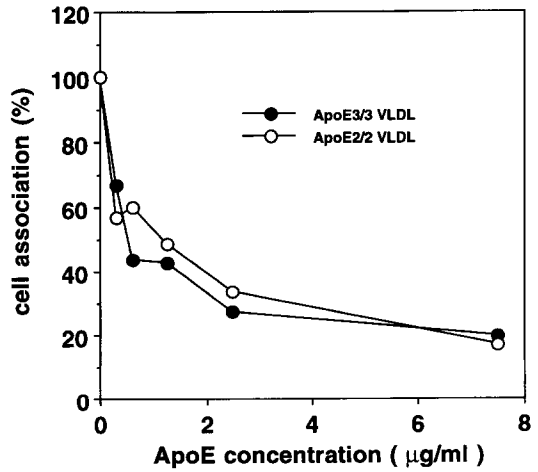

Fig. 1. Ability of the VLDL from a normolipidemic subject with two ε3 alleles and a normolipidemic subject with two ε2 alleles to compete with rabbit [^{125}I]β-VLDL for binding to VLDL receptors. Cell association (binding and internalization) of [^{125}I]lipoproteins as a function of apoE concentration. Specific activity is shown, calculated by subtracting the radioactivity associated with Neo cells from that associated with VLDL-R cells to exclude the effect of LRP. The 100% control value with no added human VLDL was 73.9 ng of [^{125}I]β-VLDL/mg of cellular protein. Each point is the average of triplicate dishes. Representative experiment.

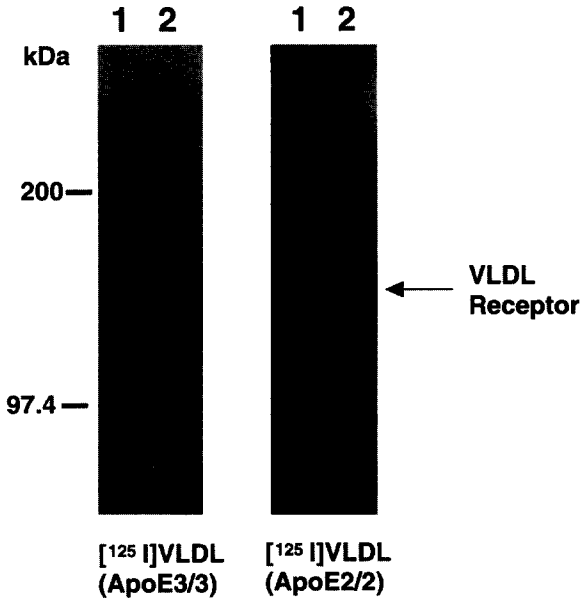

Fig. 2. Binding of human [^{125}I]VLDL of apoE3/3 and apoE2/2 to cell membrane fractions of Neo and VLDL-R cells. Membrane fractions from each transfectant were solubilized, fractionated on 7.5% SDS-polyacrylamide gels under nonreducing conditions, and transferred to a nitrocellulose filter. The filter was incubated with [^{125}I]VLDL of apoE3/3 (2.2 μg of apoE/ml, 217 cpm/ng protein) (1) or [^{125}I]VLDL of apoE2/2 (2.2 μg of apoE/ml, 220 cpm/ng protein) (2) in binding buffer containing 2 mM $CaCl_2$. A total of 7 μg of protein was loaded onto each lane. The filter was exposed to Kodak XAR5 film for 72 h at −70°C. Representative experiment.

20 μg/ml of 1D7 or 6C5 antibodies did not affect the band corresponding to the VLDL receptor blotted by [^{125}I]VLDL of apoE2/2 or [^{125}I]VLDL of apoE3/3. In the cell culture binding assay (Fig. 4), neither 1D7 nor 6C5, in doses ranging from 6.25 μg/ml to 50 μg/ml, inhibited the binding of [^{125}I]VLDL of apoE3 to the VLDL receptor. In this range, 1D7 antibody inhibited the binding of recombinant [^{125}I]apoE3 emulsified with dimyristoyl phosphatidylcholine to the human LDL receptor-overexpressing cells in a concentration-dependent manner (data not shown).

4. Discussion

The VLDL obtained from a normolipidemic subject with two ε2 alleles, as well as the VLDL obtained from a normolipidemic subject with two ε3 alleles bound to VLDL receptors with comparable affinities. Also, the anti-apoE monoclonal antibody 1D7, which inhibits binding of apoE3 to LDL receptors, failed to compete with the binding of either apoE3/3 VLDL or apoE2/2 VLDL to the VLDL receptor. These results raise the possibility that the region of the apoE molecule that binds to the VLDL receptor is not identical to the region that binds to the LDL receptor. ApoE2 has been shown to posses only 0.5–1.0% of the normal apoE3 binding activity toward the LDL receptor [14,15]. The region of apoE responsible for binding to the LDL receptor has been localized to amino acids 136–150 [5,16]. Although a striking feature of the VLDL receptor is its structural similarity to the LDL receptor, only apoE containing lipoproteins, not apoB containing LDL, bind to it. A key structural difference between the two receptors is the number of cysteine-rich repeat

sequences at the N-terminus. The VLDL receptor contains an 8-fold repeat whereas the LDL receptor consists of a 7-fold repeat. The LDL binding function of the LDL receptor requires contributions from repeats 3–7, whereas β-VLDL binding was insensitive to the loss of any single repeat, except for repeat 5 [17,18]. The addition of one more ligand binding repeat in the VLDL receptor may inhibit the binding of LDL to this receptor and makes the VLDL receptor less discriminatory against molecules other than apoE3 such as apoE2. A similar relaxed specificity toward different isoforms of human apoE has been observed in a 95-kDa oocyte-specific receptor to vitellogenin (VTG), which also binds human and rabbit apoE with high affinity [19]. This receptor was recently identified as a VLDL receptor in chicken oocyte, and termed the VLDL/VTG receptor [20].

The binding site of human apoE to the LDL receptor contains clusters of positively charged and hydrophobic residues (residues 143–150, Lys-Leu-Arg-Lys-Arg-Leu-Leu-Arg). All of these residues lie on helix 4, one of five helices located in the N-terminal domain of apoE [21]. The regions of human apoB-100 that contain such clusters have been implicated in binding to the LDL receptor (residues 3359–3368, Arg-Leu-Thr-Arg-Lys-Arg-Gly-Leu-Lys-Leu) [22,23]. Furthermore it has been shown that two such clusters of VTG, residues 493–498 (Leu-Lys-Arg-Ile-Leu-Lys) and residues 1079–1084 (Lys-Leu-Lys-Arg-Ile-Leu), mediate binding to the VLDL/VTG receptor. It is conceivable that the binding site of apoE to the VLDL receptor contains such clusters [24]. The N-terminal portion of helix 4 contains such clusters, including Arg^{134}, Arg^{136}, His^{140}, Arg^{142}, Lys143, Arg^{145}, Lys^{146}, Arg^{147}, and Arg^{150}. Because the 1D7 epitope is shown to be most likely localized in the immediate vicinity of residues 145 and 146 [5], the binding site of human apoE to the VLDL receptor might be residues 132–144. Direct binding experiments using synthetic peptides of apoE are planned to determine the binding site of apoE to the VLDL receptor. Very recently it has been shown that the VLDL receptor binds to and mediates the catabolism of LPL and urokinase complexed with plasminogen activator-inhibitor type I [25]. It raises the possibility that LPL, which would be present in the prepared VLDL fraction, may participate in the binding of VLDL to the VLDL receptor. But it seems to be unlikely because plasma LPL concentrations of subjects K.M. (apoE3/3) and N.Y. (apoE2/2) without heparin infusion were not detectable (<25 ng/ml) by enzyme immunoassay (MARKIT-F LPL, Dainippon, Tokyo, Japan).

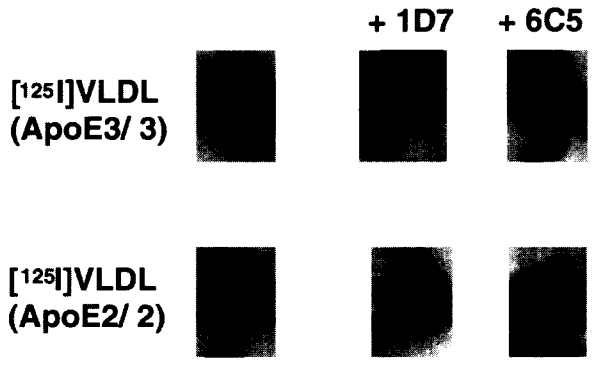

Fig. 3. The effect of apoE monoclonal antibodies on the binding of [^{125}I]VLDL to cell membrane fractions of VLDL-R cells. Nitrocellulose filters containing membrane fractions of VLDL-R cells were prepared as described in the legend to Fig. 2. Replicate filters were blotted in the presence of [^{125}I]VLDL of apoE3/3 (2.5 μg of apoE/ml, 859 cpm/ng protein) (top) or [^{125}I]VLDL of apoE2/2 (2.5 μg of apoE/ml, 654 cpm/ng protein) (bottom). [^{125}I]VLDL was preincubated with 20 μg/ml of 1D7 (+1D7) or 6C5 (+6C5) for 1 h at room temperture and then used for blotting.

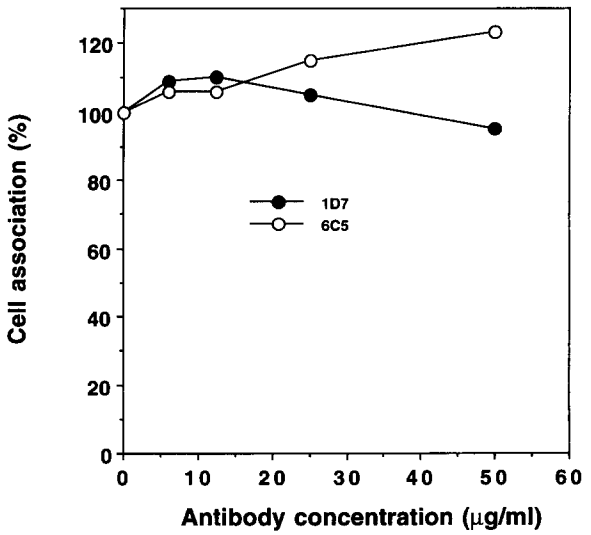

Fig. 4. The effect of apoE monoclonal antibodies on the binding of [^{125}I]VLDL to VLDL-R cells. [^{125}I]VLDL of apoE3/3 (780 cpm/ng of protein) was preincubated with the indicated concentrations of each antibody for 1 h at room temperture and then added to the wells. Final concentration of [^{125}I]VLDL was 10 μg protein/ml. Then the cells were incubated for 4 h at 37°C, and the amount of [^{125}I]VLDL that bound to the cells was determined. The specific activity is shown, calculated by subtracting the radioactivity associated with Neo cells from that associated with VLDL-R cells to exclude the effect of LRP. The 100% control value with no added antibodies was 68 ng of [^{125}I]VLDL/mg of cellular protein. Each point is the average of triplicate dishes. Representative experiment.

The scavenger receptor LRP and the VLDL receptor are not down-regulated by sterols and are candidate receptors for foam cell formation. Human VLDL obtained from fasted subjects has a significantly reduced ability to bind to the VLDL receptor compared with rabbit β-VLDL. But apoE and LPL, which are secreted from macrophages, enhanced the binding of human VLDL to the VLDL receptor [12], supporting the hypothesis that the VLDL receptor pathway may contribute to foam cell formation.

VLDL from patients homozygous for apoE2 or apoE3 bound the human VLDL receptor identically. It is possible that the VLDL receptor may contribute to atherosclerosis thorough foam cell formation, especially in patients with type III hyperlipoproteinemia.

Acknowledgements: We thank Dr. Yves Marcel for providing monoclonal antibodies directed against apoE, 1D7 and 6C5, Dr. Monty Krieger for providing ldlA-7 cells, Drs. Yutaka Kawarabayasi and Juro Sakai for helpful discussion and Naoyo Yamaguchi for technical assistance. This work was supported in part by grants-in-aid for Scientific Research from the Ministry of Science, Education, and Culture of Japan, the Ministry of Health and Welfare.

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