Apolipoprotein (apo) E genotype and apoE concentration determine binding of normal very low density lipoproteins to HepG2 cell surface receptors

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Abstract

The clinical relevance of apoE concentration in lipoprotein fractions should be evaluated. We investigated the impact of the common apolipoprotein (apo) E polymorphism in conjunction with very low density lipoprotein (VLDL) apoE concentration on the receptor binding properties of VLDL preparations from 17 normolipidemic subjects to the HepG2 cell surface receptors. All six apoE genotypes were studied. When apoE genotype alone was considered, two subgroups could be distinguished: VLDL without apoE isoform E2 (VLDL3/3, VLDL-3/4, and VLDL4/4) showed significantly higher affinity than VLDL with apoE2 (VLDL-4/2, VLDL-3/2, and VLDL-2/2). Once we adjusted for VLDL apoE content, we observed that VLDL affinity to HepG2 cell surface receptors decreased, according to apoE genotype, in the following order: VLDL4/4 (100%) > VLDL-3/4 (93%) > VLDL-2/2 (30%). Moreover, we found that VLDL apoE concentration could modify isoform-specific binding. An analysis in 47 subjects showed that the concentration of total VLDL protein and the VLDL apoE concentration varied considerably. The variation of VLDL apoE was independent of apoE genotype and corresponding serum apoE levels. We conclude that, in addition to apoE genotype, apoE content of VLDL is an important determinant of the receptor binding properties of VLDL. Bohnet, K., T. Pillot, S. Visvikis, N. Sabolovic, and G. Siest. Apolipoprotein (apo) E genotype and apoE concentration determine binding of normal very low density lipoproteins to HepG2 cell surface receptors. J. Lipid Res. 1996. 37: 1316-1324.

Supplementary key words apolipoprotein E polymorphism • apolipoprotein E concentration • hepatic receptors • binding • very low density lipoproteins

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Apolipoprotein (apo) E is a polymorphic protein that exists as three common isoforms (E3, E4, E2). The structural differences result from amino acid substitutions at residues 112 and 158. Isoform E3 has cysteine at residue 112 and arginine at residue 158, isofrom E2 has cysteine, and E4 has arginine at both sites (1). The apoE polymorphism has been shown to influence plasma concentrations of total cholesterol, low density lipoprotein (LDL) cholesterol, apoB, and apoE. Individuals carrying the e2 allele have lower cholesterol levels, and individuals carrying the e4 allele have higher cholesterol levels than those carrying the e3 allele. Sing and Davignon (2) estimated the genetic impact of apoE polymorphism on total plasma cholesterol variance to be 8.3% in a population of Ottawa, Canada. In our laboratory, using data from a population from Nancy, France, it was estimated to be 8.7% (3). An association between apoE polymorphism and cardiovascular disease has been documented (4). More recently, a linkage between apoE polymorphism and Alzheimer’s disease has been established (5-7). Furthermore, apoE has a number of other functions in man, such as immunoregulation (8) and nerve regeneration (9).

The mechanisms underlying apoE isofrom specific effects are not yet sufficiently elucidated. One important feature of apoE is its ability to serve as a ligand for different receptors. Five receptors for which apoE can serve as a ligand have been described (10): the LDL receptor, the LDL receptor related protein, the scavenger receptor, the very low density lipoprotein receptor, and the lipolysis-stimulated receptor.

The present study was undertaken to further investigate the binding of normal VLDL to the hepatic receptors. We used a new approach which aimed at simulating...
the physiological situation in normal subjects, using naturally occurring, unmodified VLDL as ligands.

We set out to determine whether there exists a direct relation between apoE polymorphism and the receptor binding properties of VLDL from normolipidemic subjects, having one of the six genotypes, and whether physiological variations of apoE concentration in VLDL influence their affinity for the hepatic receptors.

We chose HepG2 cells, a hepatoma cell line, as a model as they are known to express a wide variety of liver-specific metabolic functions, such as expression of normal LDL receptor and LDL receptor related protein (LRP), and internalization and metabolism of chylomicrons, VLDL, LDL, and high density lipoproteins (11).

MATERIAL AND METHODS

Blood was collected on EDTA from subjects coming to the Centre de Médecine Préventive in Nancy, France for health screening. ApoE genotyping was performed as described by Hixson and Vernier (12). All subjects had fasting plasma cholesterol below 7 mmol/l, triglycerides below 1.5 mmol/l, HDL-cholesterol between 0.8 and 2 mmol/l, apoA-I between 1.0 and 2.7 g/l, apoB between 0.5 and 1.3 g/l, and Lp[a] below 0.7 g/l. Plasma was separated by centrifugation and stored at 4°C. VLDL were isolated the same day by ultracentrifugation at 38,000 rpm using a Beckmann 50.3 Ti rotor. VLDL from 47 individuals were used for compositional analysis; 17 out of the 47 samples were used for binding experiments. Concentration of total proteins was determined by the method of Lowry et al. (13). Cholesterol and triglycerides were determined using standard enzymatic methods. Concentration of apoE was determined by a sandwich ELISA using polyclonal antibodies (from L. Havekes, Gaubius Laboratory, Leiden, The Netherlands), and apoB concentration by nephelometry using a Behring nephelometer. ApoC-III was estimated by an electroimmunodiffusion assay (Sebia). Radioiodination of VLDL with 125I was performed using Iodobeads (Pierce) according to the supplier’s recommendations. The specific activity of the 125I-labeled VLDL preparations ranged from 100 to 300 cpm/ng of protein. Iodinated VLDL were sterilized by filtration through 0.22-μm Millex GV filters, stored at 4°C, and used within 10 days. The human hepatoma cell line HepG2 was grown in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml fungizone, and 10% (v/v) fetal calf serum. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂. For binding experiments the cells were seeded in six-well dishes at a density of 800,000 cells per well. Twenty-four h before the experiments, fetal calf serum was replaced either by 10% (v/v) human lipoprotein-deficient serum (LPDS) or 10% (v/v) complete human serum (CHS) in order to up-regulate or down-regulate the LDL receptor expression, respectively. Before the addition of VLDL, culture medium was replaced by ice-cold serum-free RPMI 1640 medium. The cells were chilled on crushed ice for 15 min. Various amounts of radiolabeled VLDL were added to the medium and the cells were incubated for 2 h at 4°C. Nonspecific binding was determined as the binding of 125I-labeled VLDL in the presence of a 50-fold excess of unlabeled homologous VLDL. Specific binding was calculated as the difference between total and nonspecific binding. To stop the binding reaction, medium was removed and the cells were washed twice with 1 ml of ice-cold phosphate-buffered saline containing 0.2% (w/v) bovine serum albumin and twice with 1.5 ml of ice-cold phosphate-buffered saline. Cells were solubilized overnight in 0.5 M NaOH for measuring radioactivity and for determining the concentration of cellular protein.

All results are means of duplicate or quadruplicate measurements obtained by two or four independent experiments. Coefficients of variation were CV < 10% within series and CV < 15% between series. The values are expressed as ng of total VLDL protein bound/ng cell protein × 2 h. Affinity constants (Kₐ) and number of binding sites (B_max) were calculated according to Scatchard (14). Statistical analyses were performed using BMDP Statistical Software.

RESULTS

In the first series of experiments, we studied the binding of 11 VLDL preparations (VLDL-2/2A, VLDL-3/3A to C, VLDL-4/4A and B, VLDL-3/2A and B, VLDL-4/3A and B, and VLDL-4/2), to the surface of either CHS- or LPDS-treated HepG2 cells. Binding curves, as a function of total VLDL protein, are shown in Fig. 1 and 2. We observed some specific binding of all VLDL samples to CHS-treated cells with little difference between the samples, apart from a tendency of apoE2-containing VLDL (VLDL-2/2, VLDL-3/2, and VLDL-4/2) to bind somewhat less than those not containing apoE2 (Fig. 1). When cells had been preincubated with LPDS, the difference between apoE2-containing VLDL and the other samples became more obvious: binding of apoE2-containing VLDL changed very little, whereas binding of apoE3 and/or apoE4-containing VLDL increased considerably (Fig. 2).

In order to evaluate the changes in binding caused by up-regulation of the LDL receptor (preincubation with LPDS), we compared maximum binding of the samples...
to either CHS- or LPDS-treated cells. Up-regulation of the LDL receptor expression increased maximum binding of VLDL-2/2, VLDL-3/2, VLDL-4/2, VLDL-3/3, VLDL-3/4, and VLDL-4/4 by a factor of 1.24 (n = 1), 1.27 (n = 2), 1.63 (n = 1), 2.54 ± 0.50 (mean ± SD; n = 3), 2.24 ± 0.12 (n = 2), and 2.45 ± 0.02 (n = 2), respectively. We distinguished two subgroups: preincubation of cells with LPDS enhanced the maximum binding of VLDL-4/4, VLDL-3/4 and VLDL-3/3 significantly more than the binding of VLDL-4/2, VLDL-3/2 and VLDL-2/2 (P < 0.01).

Data from Fig. 2 were transformed according to Scatchard (14), in order to calculate affinity constants (K_d) and number of binding sites (B_max). We again identified two subgroups: VLDL-3/3, VLDL-4/4, and VLDL-4/3 showed significantly greater affinity to HepG2 cell surface than VLDL-2/2, VLDL-3/2, and VLDL-4/2 (P < 0.02). As shown in Table 1, VLDL-3/3A had the highest affinity (K_d = 6.2 μg/ml), and highest maximum binding, which we set equal to 100%. VLDL-2/2A had the lowest affinity and maximal binding was only 35% of that of VLDL-3/3A. The number of binding sites (B_max) was significantly lower for VLDL containing apoE2, than for VLDL containing only apoE3 and/or apoE4 (P < 0.01).

Among the different samples of VLDL-3/3, 4/3, and 4/4, the affinity was not systematically in agreement with apoE phenotype. In order to explain these differences, we tried to relate binding of VLDL to their apoE content and not only to their content of total protein. An analysis of 47 VLDL preparations showed that their composition varied considerably. The composition of VLDL used for binding experiments is shown in Table 2.

Figure 3 shows binding curves of VLDL, as a function of VLDL apoE, to the surface of LPDS-preincubated HepG2 cells. When results were expressed as a function of VLDL apoE, VLDL having the same apoE phenotype showed a very similar degree of binding. ApoE concent-
Fig. 2. Specific binding of $^{125}$I-labeled VLDL to LPDS-treated HepG2 cells. (A) VLDL isolated from homozygous donors. (B) VLDL isolated from heterozygous donors. Before the binding experiments, HepG2 cells were incubated for 24 h with medium containing 10% (v/v) LPDS. Various amounts of radiolabeled VLDL were added to the medium and the cells were incubated for 2 h at 4°C. Nonspecific binding was determined as the binding of $^{125}$I-labeled VLDL in the presence of a 50-fold excess of unlabeled homologous VLDL. Specific binding was calculated as the difference between total and nonspecific binding. The genotype of each VLDL sample is indicated at the end of the corresponding curve [VLDL-2/2 (A), VLDL-3/3 (B), VLDL-4/4 (C), VLDL-3/2 (D), VLDL-3/4 (E), VLDL-4/2 (F)].

High amounts of apoE in VLDL could increase receptor binding and VLDL containing different apoE isoforms achieved the same binding at different apoE concentrations: a binding of 200 ng VLDL-4/4 or VLDL-3/3 was achieved at an apoE content of 62 ± 10 ng (mean ± SD, n = 4) or 112 ± 21 ng (n = 6), respectively. This difference was significant at P = 0.01 (Table 4). Under our experimental conditions, none of apoE2-containing

TABLE 1. Affinity constants ($K_d$) and maximal binding sites ($B_{max}$) from binding experiments of $^{125}$I-labeled VLDL to HepG2 cells

<table>
<thead>
<tr>
<th>VLDL ApoE</th>
<th>% of Maximal Binding Related to Total VLDL Protein</th>
<th>% of Maximal Binding Related to VLDL ApoE</th>
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<tbody>
<tr>
<td>VLDL-4/4A</td>
<td>6.7</td>
<td>484</td>
</tr>
<tr>
<td>VLDL-4/3A</td>
<td>8.6</td>
<td>494</td>
</tr>
<tr>
<td>VLDL-4/4B</td>
<td>9.5</td>
<td>465</td>
</tr>
<tr>
<td>VLDL-4/5B</td>
<td>9.5</td>
<td>551</td>
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<td>VLDL-3/5C</td>
<td>10.4</td>
<td>409</td>
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<td>6.2</td>
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</tr>
<tr>
<td>VLDL-3/5B</td>
<td>9.5</td>
<td>447</td>
</tr>
<tr>
<td>VLDL-4/2</td>
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<td>354</td>
</tr>
<tr>
<td>VLDL-5/2A</td>
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<tr>
<td>VLDL-3/2B</td>
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</tr>
<tr>
<td>VLDL-2/2A</td>
<td>21.3</td>
<td>273</td>
</tr>
</tbody>
</table>

*According to Scatchard (14).
samples achieved a binding of 200 ng $^{125}$I-labeled VLDL per mg cellular protein, even at an apoE content of 200 ng.

As shown in Table 4, we did not observe a significant relationship between the concentrations of VLDL triglycerides, apoB, and apoC-III and the amount of $^{125}$I-labeled VLDL bound to HepG2 cell surface receptors ($P > 0.05$). For cholesterol, Mann-Whitney rank-sum test revealed a difference between VLDL-4/4 and VLDL-3/3 at $P < 0.05$, which is perhaps of doubtful biological significance due to the small sample size. We also drew binding curves for VLDL as a function of VLDL total cholesterol, triglycerides, apoB, and apoC-III (data not shown). No correlation between VLDL receptor binding and VLDL total cholesterol, triglycerides, apoB, and apoC-III was observed.

When comparing binding of the different samples after adjustment for apoE content, we observed that the order from highest to lowest maximum binding had changed (Table 1). At an apoE content of 200 ng, VLDL-4/4 had the highest amount bound, when mean values were considered (VLDL-4/4A 100% and VLDL-4/4B 93%; we set the mean value, 96.5%, equal to 100% in order to evaluate the maximum binding of the other VLDL samples). Maximum binding decreased, according to apoE genotype, in the following order: VLDL-4/4 (100%) > VLDL-4/3 (93 ± 3%) > VLDL-3/3 (82 ± 2%) > VLDL-4/2 (53%) > VLDL-3/2 (36 ± 1%) > VLDL-2/2 (30%).

### DISCUSSION

The mechanisms of VLDL clearance are not yet completely understood, but there is a growing body of evidence that at least two receptors are involved in normal VLDL catabolism: the LDL receptor and the LRP (15, 16). VLDL binding to the LDL receptor seems to be mediated mainly by apoE. Bradley and Gianturco (17) compared the binding of large VLDL from hypertriglyceridemic patients and from normal subjects to human skin fibroblasts and stated that apoE is necessary and sufficient for the binding of these particles to the LDL receptor. Krul, Tikkanen, and Schonfeld (18) emphasized the importance of apoE conformation as determinant for VLDL binding to the LDL receptor, although current understanding of apoE conformation in VLDL is relatively limited (19). Eisenberg, Friedmann, and Vogel (20) added recombinant apoE3 to VLDL which caused a manyfold enhancement of their metabolism by human skin fibroblasts.

Binding of VLDL to LRP seems to involve not only apoE, but also lipoprotein lipase and heparan sulfate proteoglycans. Several studies have shown, that apoE-enriched lipoprotein particles can bind to LRP. Kowal et al. (21) showed that LRP can mediate the catabolism of lipoproteins that are enriched in apoE. Ji, Fazio, and Mahley (22) provide evidence that heparan sulfate proteoglycans are involved in the binding of apoE-rich
lipoprotein particles to LRP. They proposed that heparan sulfate proteoglycans and LRP may be operating as a complex, which would enhance binding of apoE-enriched remnant lipoproteins. Evidence, that not only apoE-enriched lipoprotein particles but also normal human VLDL can be catabolized by LRP comes from a study from Chappell et al. (23).

In vitro studies have demonstrated that the apoE polymorphism influences the receptor binding properties of apoE, which might explain some of the observed isoform specific effects. Weisgraber, Innerarity, and Mahley (24) have shown that the apoE isoforms differ in their ability to compete with human 125I-labeled LDL for binding sites on fibroblasts, the capacity of apoE2 for competing being about 1% of the capacities of apoE3 and apoE4. Kowal et al. (25) and Ji et al. (22) enriched rabbit P-VLDL with different apoEs and stated that different apoE isoforms bind differently to LRP and to heparan sulfate. Using direct binding assay, Mann et al. (26) showed that apoE2 and some functionally defect apoE variants had clearly reduced capability to mediate β-VLDL binding to LRP, probably due to reduced capacity of these forms to bind to heparin. Demant et al. (27) provide in vivo evidence that VLDL clearance is dependent on apoE genotype. Gregg and Brewer (28) proposed that the isoform specific receptor binding properties of apoE could explain the influence of apoE polymorphism on plasma cholesterol levels: due to a defective receptor binding of apoE2, internalization and catabolism of cholesterol-rich chylomicron and VLDL remnants are decreased which leads to an up-regulation of the LDL receptor expression. As a consequence, LDL are catabolized more rapidly resulting in a decrease in plasma cholesterol levels in e2 homozygotes. The opposite might occur in e4 homozygotes.

We have extended those observations here. To our knowledge this is the first study to investigate the effect of apoE polymorphism on the binding of native VLDL.

![Fig. 3. Specific binding of 125I-labeled VLDL to LPDS-treated HepG2 cells as a function of VLDL apoE. (A) VLDL isolated from homozygous donors. (B) VLDL isolated from heterozygous donors. Before the binding experiments, HepG2 cells were incubated for 24 h with medium containing 10% (v/v) LPDS. Various amounts of radiolabeled VLDL were added to the medium and the cells were incubated for 2 h at 4°C. Nonspecific binding was determined as the binding of 125I-labeled VLDL in the presence of a 50-fold excess of unlabeled homologous VLDL. Specific binding was calculated as the difference between total and nonspecific binding. The genotype of each VLDL sample is indicated at the end of the corresponding curve [VLDL-2/2 (A), VLDL3/3 (O), VLDL4/4 (C), VLDL3/2 (M), VLDL3/4 (x), VLDL4/2 (O)].](image)
to the hepatic receptors, taking into account all six apoE genotypes and physiological variations of apoE concentration in VLDL.

We observed that normal human VLDL bound differently to LPDS- and to CHS-treated HepG2 cells and that the magnitude of this difference depended on the apoE isoform present in the VLDL sample. Up-regulation of the LDL receptor led to a significant increase in number of binding sites and thus to an increase of maximum binding of VLDL containing apoE3 and/or apoE4. Maximum binding of apoE2-containing VLDL increased very little after up-regulation of the LDL receptor. These results confirm the involvement of the LDL receptor in cell capture of apoE3- and/or apoE4-containing VLDL. This is in good agreement with the results of Weisgraber et al. (24), who have shown that apoE2 has only a negligible affinity to the LDL receptor. The specific binding of apoE2-containing VLDL to LPDS- as well as to CHS-treated cells could be mediated by LRP, which is known to be present on the surface of HepG2 cells and for which apoE2 has been shown to have an affinity that is about 40% of that of apoE3 or apoE4 (25). Our results suggest that, even in LDLR-/- and/or apoE-/- containing VLDL (16) and provide evidence that this receptor is not or only little involved in the capture of apoE2-containing VLDL. This is in good agreement with the results of Weisgraber et al. (24), who have shown that apoE2 has only a negligible affinity to the LDL receptor.

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After adjustment for apoE concentration, affinity of VLDL-3/3 for HepG2 cell surface receptors was 82 ± 2% of the affinity of VLDL-4/4, that of VLDL-2/2 was 30%.

This in vitro observation is in good agreement with in vivo observations of Gregg et al. (28) who have shown that, in normal subjects, apoE4 is catabolized more rapidly and apoE2 more slowly than apoE3. ApoE-mediated processes might occur at an increased rate in e4/e4 subjects. Our findings support this hypothesis; the enhanced receptor affinity of apoE4 could be responsible for its accelerated catabolism.

We now propose that, in addition to apoE polymorphism, apoE concentration in VLDL might also regulate metabolic processes. We emphasize that only determination of apoE in lipoprotein particles and not determination of total plasma apoE might provide additional information. It has already been suggested that the determination of apoE concentration in high density lipoproteins rather than the determination of total plasma apoE concentration, provides information when evaluating an individual’s risk for developing cardiovascular disease (29) and our study suggests that apoE concentration in VLDL might also be of importance. We have shown that physiological variations in apoE concentration in VLDL are sufficient to modify their affinity to the hepatic receptors and that VLDL containing an apoE isoform with lower receptor affinity (apoE3) in higher concentration can bind to HepG2 cell surface receptors equally well as VLDL containing an apoE isoform with higher receptor affinity (apoE4) in lower concentration. High apoE concentration in VLDL might accelerate their internalization and catabolism, lead to down-regulation of the LDL receptor expression, and subsequently to elevated cholesterol levels and eventually to an increased risk for developing cardiovascular disease. However, it has been demonstrated that injection of apoE into Watanabe rabbits protects them.
against atherosclerosis (30). Experiments with transgenic mice have shown that apoE-deficient mice develop severe hypercholesterolemia and atherosclerotic lesions (31). Probably, too high as well as too low apoE concentration might disturb lipid homeostasis. Obviously, regulation of the lipid metabolism is very complex and many questions remain unanswered. The role of apoE concentration in lipoprotein particles needs to be carefully investigated.

On the basis of the results described here, we suggest that affinity of VLDL from normolipidemic subjects to the hepatic receptors is mainly regulated by apoE genotype in conjunction with VLDL apoE concentration and we conclude that it is of importance to consider VLDL apoE concentration as a parameter that could influence total plasma cholesterol level by mechanisms involving binding and metabolism of normal VLDL via specific interactions with the LDL receptor and the LRP. The clinical relevance of apoE concentration in lipoprotein fractions should be evaluated. 

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