Apolipoprotein E isoforms and rare mutations: parallel reduction in binding to cells and to heparin reflects severity of associated type III hyperlipoproteinemia

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Abstract The LDL receptor-independent binding of human apolipoprotein E isoforms and rare apoE mutations were studied on LDL receptor-deficient human fibroblasts using chemical cross-linking and cell binding studies. The cross-linking experiments demonstrated that all apoE variants bind to the low density lipoprotein receptor-related protein, a potential receptor for remnant lipoproteins. In cell binding studies, the effect of the apoE variants on binding of β-VLDL was investigated. Addition of normal apoE-3 to the binding assay resulted in a 12-fold increase of β-VLDL particle binding, whereas this effect was reduced in the clinically defective variants: apoE-2, ($Arg^{158}\rightarrow Cys$), 24.4% of apoE-3; apoE-1, ($Gly^{127}\rightarrow Asp$, $Arg^{158}\rightarrow Cys$), 49.2% of apoE-3; apoE-1($Lys^{146}\rightarrow Glu$), 18.2% of apoE-3. Heparin binding studies with the same variants showed a parallel reduction in proteoglycan binding (apoE-2^{158}, 58.2% of apoE-3; apoE-$1^{127,158}$, 37.9%; apoE-1^{146}, 20.6%). We conclude that LDL receptor-independent mechanisms contribute to remnant clearance. The functionally dominant mutation apoE-1^{146} was most defective in heparin binding studies in vitro. In cell binding studies, apoE-1^{146} did mediate lipoprotein binding only 18% compared to apoE-3. This indicates the important role of the apoE interaction with proteoglycans in vivo and could explain the development of type III hyperlipoproteinemia in patients with such apoE variants.—**Mann, W. A., N. Meyer, W. Weber, S. Meyer, H. Greten, and U. Beisiegel.** Apolipoprotein E isoforms and rare mutations: parallel reduction in binding to cells and to heparin reflects severity of associated type III hyperlipoproteinemia. *J. Lipid Res.* 1995. **36:** 517–525.

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Apolipoprotein E (apoE) is a protein constituent of triglyceride-rich chylomicron and VLDL remnants in plasma and mediates their catabolism by interacting with receptors on cell surfaces (1). The low density lipoprotein (LDL) receptor contributes to the metabolism of these apoE-containing particles (2), but there is evidence for additional LDL receptor independent mechanisms (3). Recently, the low density lipoprotein receptor-related protein (LRP) has been shown to be an apoE binding protein (4–6) in vitro. However, the in vivo significance of the apoE-LRP interaction has not yet been clarified, even though a recent publication indicates an in vivo role of LRP in chylomicron remnant clearance (7).

A disease characterized by delayed chylomicron and VLDL remnant catabolism is type III hyperlipoproteinemia (HLP), which is most frequently associated with homozygosity for the apoE-2($Arg^{158}\rightarrow Cys$) allele (8). ApoE-2^{158} has been found to be functionally defective both in vitro in binding to the LDL receptor (9) and in vivo in delayed catabolism from plasma (10). The fact that only 1–5% of homozygous carriers of the apoE-2 allele develop a clinical manifestation of disease (11) indicates that other hyperlipidemic factors (environmental or genetic) need to contribute to the development of type III HLP. Thus, in most patients with type III HLP, the presence of apoE-2 is necessary but not sufficient for remnant accumulation and apoE-2 is therefore associated with type III HLP in a recessive mode. However, the presence of some rare mutations of apoE results in a dominant expression of type III HLP. In the affected families, one mutant apoE allele was sufficient for the development of type III HLP. To date five dominant mutations have been described: apoE-2($Lys^{146}\rightarrow Gln$) (12, 13), the double mutant apoE-3($Cys^{112}\rightarrow Arg$, $Arg^{142}\rightarrow Cys$) (14, 15), apoE-3_{Leiden} ($Cys^{112}\rightarrow Arg$, duplication of residues 120–126 or 121–127) (16–18), apoE-$4_{Philadelphia}$

Abbreviations: SADP, N-succinimidyl(4-azidophenyl) 1,3'-dithiopropionate; EDC/NHS, 1-ethyl-3(3-dimethylaminopropyl) carbo-diimide/N-hydroxysulfosuccinimide; alpha 2-MR, alpha 2 macroglobulin receptor; LRP, low density lipoprotein receptor-related protein; FH, familial hypercholesterolemia; HLP, hyperlipoproteinemia; FCS, fetal calf serum.

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(Glu^{13}→Lys, Arg^{145}→Cys) (19, 20), and apoE-1$_{Harrisburg}$ (Lys^{146}→Glu) (21–23). LDL receptor affinity of these mutations ranged from 7.7–40% compared to the activity of apoE-3 (13, 22–24) and in no case was the defect in binding to the LDL receptor more severe than the defect observed for apoE-2(Arg^{158}→Cys). Thus, differential affinity of apoE mutations to the LDL receptor seems not to be the discriminating factor between recessive or dominant expression of type III hyperlipoproteinemia.

We therefore investigated LDL receptor independent binding of apoE-3, apoE-2(Arg^{159}→Cys) and two mutations apoE-1(Gly^{127}→Asp, Arg^{158}→Cys) and apoE-1(Lys^{146}→Glu). ApoE-1^{127,158} is an example of a functionally recessive mutation (25), whereas apoE-1^{146} is dominantly associated with type III hyperlipoproteinemia.

We analyzed the binding of these mutants to heparin as a model for proteoglycan binding. The binding to cell surface receptors was studied by chemical crosslinking of apoE to its receptor. In addition, the effect of the apoE mutants on β-VLDL binding to LDL receptor-deficient fibroblasts (FH-fibroblasts) and human hepatoma (Hep3B) cells was analyzed. With these studies we wanted to find out whether binding defects of apoE mutants to proteoglycans or LRP may reflect the severity of the clinically associated type III HLP.

METHODS

Apolipoprotein E and A-I isolation

ApoE-1^{146} was isolated from a patient with the apoE-1/3 phenotype by preparative ultracentrifugation (26) followed by delipidation with chloroform–methanol 2:1. Heparin affinity chromatography was used to separate apoE-1^{146} from apoE-3 and the other apolipoproteins (27). ApoE-1(Gly^{127}→Asp, Cys^{158}→Arg), apoE-2, and apoE-3, apoE-3/4, and apoA-I were isolated from patients homozygous (heterozygous) for the respective apoE alleles by ultracentrifugation followed by preparative SDS-polyacrylamide gel electrophoresis (13%). Quality of the respective apoE preparation was documented by analytical SDS-PAGE (data not shown). The ability to bind to LDL receptors on normal human fibroblasts was measured by the displacement of ^{125}I-labeled LDL by apoE-phospholipid complexes (liposomes). ApoE liposomes were prepared as described by Havinga, Lohse, and Beisiegel (28). Briefly, 25 μg apoE in 100 μl 50 mM borate buffer, pH 8, with 2.34 mg sodium cholate was added to a lipid film, consisting of 0.2 mg egg lecithin and 4 μg cholesterol. The solution was mixed and dialyzed against borate buffer for 48 h at 4°C. For the crosslinking experiments, apoE was iodinated by the iodogen method (29) before introduction into liposomes. The iodination efficiency ranged from 20 to 50%; specific activities for apoE ranged between 300 and 600 cpm/ng of protein.

β-VLDL, LDL

Rabbit β-VLDL (d < 1.006 g/ml) and human LDL were isolated from cholesterol-fed rabbits (30) and normolipidemic patients (blood donors), respectively. Iodination was determined by the iodine monochloride method (31). Specific activities ranged from 70 to 100 cpm/ng protein.

Reassociation of apoE with β-VLDL

To compare the ability of the different apoE isoforms to reassociate with β-VLDL, β-VLDL (10 μg) was incubated with ^{125}I-labeled apoE-3 or the respective isoform (80 μg) in PBS (0.5 ml) for 30 min at 37°C followed by ultracentrifugation (d 1.019 g/ml, total volume 1 ml). The top fraction (100 μl) was collected and the radioactivity was measured to determine the amount of apoE reassociated with the lipoproteins. To compare the effect of reassociated apoE with apoE added directly to the medium, different concentrations of apoE-3 (10–160 μg) were reassociated with β-VLDL (10 μg/ml) as described above and reisolated by ultracentrifugation. The d 1.019 g/ml top fraction containing the lipoproteins was used directly for the binding assays.

Analytical methods

ApoE phenotypes were determined by analytical isoelectric focusing gel electrophoresis (32). Cholesterol and triglyceride levels were measured with enzymatic colorimetric assays from Boehringer Mannheim. Protein determinations were done by the method of Lowry et al. (33).

Antibodies

Polyclonal anti-LRP/alpha-2 MR antibody was obtained from J. Gliemann (Aarhus, Denmark). The preparation and characterization of this antibody has been described previously (34). As detection antibody a peroxidase-labeled polyclonal goat anti-rabbit antibody (Jackson Biochem.) was used.

Cells

Human hepatoma cells (Hep3B) and normal human fibroblasts were maintained in DMEM (GIBCO), 10% FCS, 0.1% penicillin/streptomycin at 37°C in 5% CO_2. LDL receptor-deficient fibroblasts were a gift from J. Davignon (Montreal, Canada). The LDL receptor defect consists of a > 10 kilobase deletion of the promoter and first exon of the LDL receptor gene (35). Fibroblasts were grown under the same conditions as the hepatoma cells.

Cell binding studies

On day 0 the cells (ca. 1 × 10^6) were placed in 24-well plates (Costar, Cambridge, MA) and maintained in growth medium. On day 2, the medium was replaced with DMEM, 5% BSA, and ^{125}I-labeled β-VLDL as indicated in the figure legends. Apolipoproteins E or A-I were added directly to the incubation mixture except in the ex-

periments indicated. After incubation for 40 min at 4°C, media were collected, cells were washed 6 times with ice-cold PBS/0.2% BSA, and the bound particles were released with heparin (5 mg/ml, 153.5 U/mg, Hoffmann-La Roche). The cells were lysed with 0.1 N sodium hydroxide. Cell-associated radioactivity was quantitated in an aliquot of the lysed cells; another aliquot was used for cell protein determination. Bound or cell associated particles were expressed as ng of ligand/mg cell protein.

LDL displacement by apoE

To directly determine the LDL (apoB,E) receptor affinity of the apoE variants, their ability to displace radioiodinated LDL from receptors on normal human fibroblasts was quantitated (36). ApoE–phospholipid complexes (apoE-liposomes) were used as competitors and prepared as described above. After up-regulation of LDL receptors by incubation in 10% LPDS for 48 h, the cells were chilled to 4°C for 20 min. The medium was replaced with medium containing ^{125}I-labeled LDL (2.5 μg protein/ml) and apoE-liposomes in concentrations of 1, 2, 4, 8 μg/ml. Cells were incubated for 4 h at 4°C, the cell supernatant was collected, and the cells were washed and lysed as above. Cell associated radioactivity was quantitated by trichloroacetic acid precipitation of an aliquot of the lysed cells. Cell protein was determined and cell-associated LDL was calculated per μg cell protein. The competition data were used to calculate the apoE-liposome concentration required to displace 50% of bound LDL. The concentration of normal apoE-3 to displace 50% of bound LDL was defined as 100%.

Crosslinking experiments

Binding was performed for 90 min at 4°C using ^{125}I-labeled apoE liposomes in 10-cm Petri dishes in 5 ml DMEM. After a final wash with PBS (pH 7.0) the bound ligand was chemically linked to its receptor by the crosslinker 1-ethyl-3(3-dimethylaminopropyl) carbodiimide in combination with N-hydroxysulfosuccinimide (Pierce). Both reagents were used at a final concentration of 1 mg/ml in PBS at pH 7. Incubation with the crosslinking reagents lasted 30 min, followed by a wash with PBS. Subsequently the cells were harvested from the dishes, spun at 400 *g* for 10 min, and lysed with 20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 2 mM MgCl, 1% Nonidet NP-40. Insoluble proteins were removed by centrifugation in a TL-100.2 rotor (Beckman) for 10 min at 55,000 RPM and NP-40-soluble proteins were separated by 5% SDS-PAGE. Gels were electroblotted to nitrocellulose (0.45 μm, Schleicher & Schuell), which was exposed for autoradiography.

The position of LRP was located by immunostaining with a polyclonal antibody, which was provided by J. Gliemann (Aarhus, Denmark). The specific activities of the apoE isoforms were as follows: apoE-1^{146}: 472 dpm/ng, apoE-2: 463 dpm/ng, apoE-3: 302 dpm/ng, apoE3/4: 574 dpm/ng.

Heparin binding

Heparin-Sepharose and normal Sepharose CL6B were obtained from Pharmacia (Piscataway, NJ) and prepared as recommended by the manufacturer. For binding of apoE, 4 mg of heparin-Sepharose or 4 mg Sepharose was incubated for 4 h at 4°C on an overhead shaker in 200 μl 20 mmol Tris/HCl, pH 7.5, 50 mmol NaCl, and 1% BSA containing 0.5 μg protein/ml of radiolabeled apoE. After the incubation the gel matrix was sedimented by centrifugation (10,000 *g*). The radioactivity in 100-μl aliquots of the top and bottom fractions was quantitated and the gel-bound ligand was calculated. Heparin-bound ligand was corrected for Sepharose binding by subtraction of Sepharose gel-bound radioactivity and expressed in ng apoE bound per μg heparin. The mass of heparin in heparin-Sepharose was quantitated by uronic acid determination (37).

RESULTS

The binding characteristics of normal apoE-3 and the variants were first examined by measuring their ability to interact with LDL receptors on normal human fibroblasts. In a competition assay apoE liposomes were used to displace ^{125}I-labeled LDL: for 50% displacement of LDL binding, 68.7 ± 7.1 ng/ml of normal apoE-3 was required, which compares well with published data (38) indicating that the apoE remained functionally intact during the isolation procedure.

The clinically defective variants showed a reduction in LDL-receptor binding ranging from 7.7 to 10.6% (apoE-1^{146}: 7.7 ± 0.7%; apoE-1^{127,158}: 10.6 ± 1.1%; apoE-2^{158}: 8.5 ± 1.0%) of the activity of normal apoE-3.

These results indicate that the reduction in LDL receptor binding does not correlate with dominant manifestation of type III HLP. Thus LDL receptor interactions were omitted by using FH-fibroblasts.

The interaction of the apoE isoforms and mutants with FH-fibroblasts was investigated on a qualitative level with crosslinking studies. We compared the binding of different ^{125}I-labeled apoE liposomes (apoE-1^{146}, apoE-2, apoE-3, and apoE-3/4) using the chemical crosslinker 1-ethyl-3(3-dimethylaminopropyl) carbodiimide/N-hydroxysulfosuccinimide and ^{125}I-labeled apoE isoproteins and mutants. One major aim of this experiment was to evaluate whether the functionally dominant mutation apoE-1^{146} would be qualitatively deficient in its ability to bind to LRP when compared to the functionally recessive apoE-2. A 600-kd band labeled by bound apoE could be obtained for all cases, indicating that apoE-1^{146}, apoE-2, apoE-3, and apoE-3/4 are principally able to bind to LRP

(**Fig. 1**). Identity of this band with LRP was shown using a polyclonal antibody against LRP. The bands obtained for apoE-1^{146} and apoE-2 appeared less extensive than the ones obtained for apoE-3 and apoE-3/4, but given the complexity of the procedure a quantitative conclusion is not indicated.

In order to quantitate the cell binding properties of the different apoE isoproteins we studied FH-fibroblasts and a human hepatoma cell line (Hep3B). We used β-VLDL as model for remnant particles, which has been shown to be taken up by LRP (5).

First, the ability of apoE isoforms and mutants to reassociate with β-VLDL was compared. Incubation of β-VLDL with radioiodinated apoE-3, E-2^{158}, E-1^{146}, and apoE-1^{127,158} showed that 38–40.6% of the respective apoE could be recovered with the lipoproteins after ultracentrifugation (d 1.019 g/ml). Thus the ability to reassociate with β-VLDL was comparable for all apoE variants used in the subsequent cell binding studies.

Next, we compared the effect of apoE associated with β-VLDL to the effect of adding apoE directly to the cell medium. A dose-dependent effect of increasing amounts of apoE-3 on the ^{125}I-labeled β-VLDL binding to Hep3B cells (**Fig. 2A**) was seen. In this experiment apoE was incubated with ^{125}I-labeled β-VLDL followed by reisolation of the β-VLDL/apoE complex by ultracentrifugation.

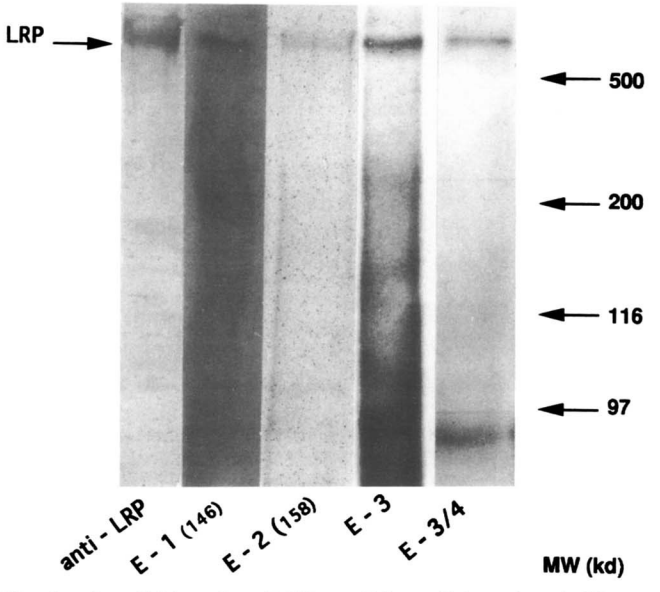

Fig. 1. Crosslinking of apoE-1^{146}, apoE-2, apoE-3, and apoE-3/4 to LDL receptor-deficient FH-fibroblasts. Fifteen μg ^{125}I-labeled apoE liposomes (E-1^{146}Lys→Glu, E-2, E-3, E-3/4) were incubated with FH-fibroblasts for 90 min at 4°C. Bound apoE was chemically crosslinked to its receptor using EDC/NHS (1 mg/ml). Cell proteins were solubilized with a Nonidet P-40-containing buffer, followed by centrifugation to remove unsolubilized proteins. The supernatants were separated by 5% SDS-PAGE, electroblotted to nitrocellulose, and exposed for autoradiography for ca. 7 days. Lane 1 shows the position of LRP by immunostaining with an polyclonal anti-LRP/ alpha 2-MR antibody, lane 2 the band obtained with apoE-1^{146}, lane 3: apoE-2, lane 4: apoE-3, lane 5: apoE-3/4.

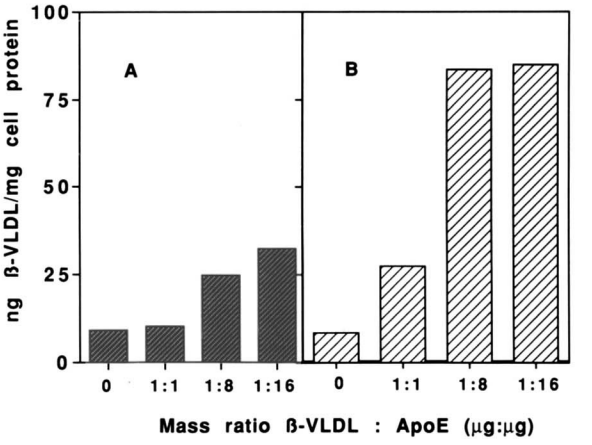

Fig. 2. Effect of apoE-3 on binding of β-VLDL to Hep3B cells: comparison of reassociation versus direct addition of apoE. Hep3B cells (1×10^6) were incubated with ^{125}I-labeled β-VLDL (1 μg/ml) with increasing amounts of apoE-3 for 1 h at 4°C. The effect of reassociating apoE with β-VLDL was compared to the direct addition of apoE to the assay medium. For the reassociation experiments ^{125}I-labeled β-VLDL was incubated with increasing amounts of apoE-3 (mass ratio: 1:1, 1:8, 1:16) for 30 min at 37°C. Unbound apoE was removed by ultracentrifugation (d 1.019 g/ml) and the ^{125}I-labeled β-VLDL/apoE complex was used for the binding assay. Panel A shows the specific binding of ^{125}I-labeled β-VLDL (1 μg/ml) reassociated with apoE at the indicated mass ratios. Panel B shows the specific binding of ^{125}I-labeled β-VLDL with apoE directly added to the assay medium at the indicated mass ratios. Nonspecific binding was measured in the presence of a 50-fold excess unlabeled β-VLDL. Values represent the mean of duplicate determinations (SD < 15%).

Thus free apoE was removed before the cell binding assay. The effect of increasing amounts of apoE-3 added directly to the cell medium is shown in Fig. 2B. In this experiment apoE was allowed to reassociate with ^{125}I-labeled β-VLDL in the assay medium. In addition, apoE could also bind to the cell surface or move freely in the assay medium. Specific binding of β-VLDL is more efficiently increased by direct addition of apoE (maximum binding: 84.9 ng/mg cell protein) than by β-VLDL/apoE (32.2 ng/mg cell protein). Because there was a more pronounced effect of direct addition of apoE we used this method for the subsequent cell binding studies.

Figure 3 shows the dose–response curve obtained by addition of increasing amounts of apoE-3 to β-VLDL on Hep3B cells. Addition of apoE-3 resulted in an increase of total binding from 20.1 up to 129.1 ng/mg cell protein. Nonspecific binding was measured after suppression with a 50-fold excess of unlabeled β-VLDL and was < 20% of total binding (Fig. 3). The increase in β-VLDL binding mediated by apoE-2 and apoE-3 is shown in **Figure 4.** A concentration-dependent increase in specific binding can be observed. Whereas the maximum binding achieved with apoE-3 is 117 ng/mg, addition of apoE-2 results in a maximum increase to 42 ng/mg cell protein (35.9% of apoE-3).

Though little LDL receptor activity should be present

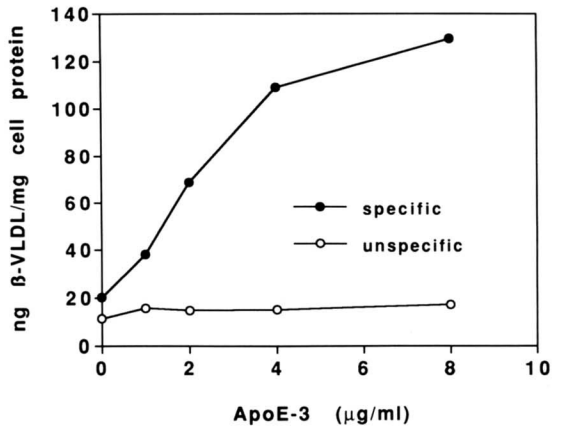

Fig. 3. Effect of apoE-3 concentrations on binding of β-VLDL to Hep3B cells. Hep3B cells (1×10^6) were incubated with ^{125}I-labeled β-VLDL (1 µg/ml) with increasing amounts of apoE-3 for 1 h at 4 °C. Addition of apoE-3 resulted in an increase of specific binding from 20.5 ng/mg cell protein to 129.1 ng/mg (●). Nonspecific binding was measured in the presence of a 50-fold excess unlabeled β-VLDL and remained below 20 ng/mg (○). Values represent the mean of duplicate determinations (SD < 15%).

in the unstimulated hepatoma cells (no LDL-receptor stimulation with cholesterol-free medium), FH-fibroblasts were used to exclude the influence of LDL receptors. Again a dose-dependent effect of apoE on β-VLDL binding could be observed with the nonspecific binding ranging from 17.5 to 19.8%. To exclude nonspecific protein/protein interactions we added apoA-I instead of apoE-3 to the incubation, which did not affect β-VLDL binding (**Fig. 5**).

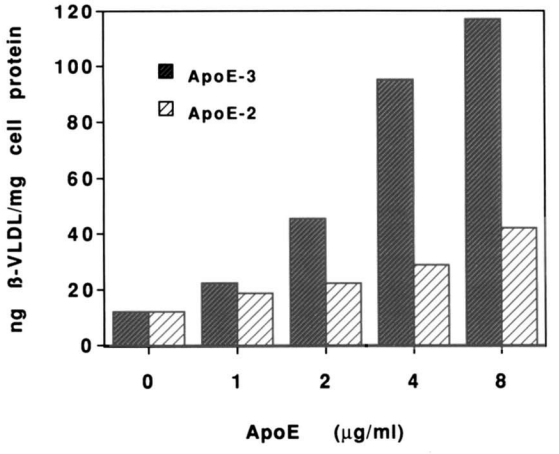

Fig. 4. Binding of β-VLDL to Hep3B cells: differential effect of apoE-2 and apoE-3. Hep3B cells (1×10^6) were incubated with ^{125}I-labeled β-VLDL (1 µg/ml) in the absence or presence of increasing amounts of apoE-2 or apoE-3 for 1 h at 4°C. Addition of apoE-3 up to 8 µg/ml increased specific binding of β-VLDL 14-fold. The effect of apoE-2 to mediate β-VLDL binding was reduced to 36% compared to apoE-3. Nonspecific binding was subtracted after suppression of binding with a 50-fold excess unlabeled β-VLDL. Bound ligand is expressed in ng/mg cell protein. Values represent the mean of three independent experiments (SD < 15%).

Having established a dose-dependent, specific effect of normal apoE-3 in both Hep3B and FH-fibroblasts, we now compared the effect of apoE-1^{146}, apoE-$1^{127,158}$, apoE-2^{158}, and normal apoE-3 on β-VLDL binding on FH-fibroblasts. Without the addition of apoE, the basal specific binding for β-VLDL was 4.5 ng/mg cell protein. Addition of normal apoE-3 resulted in an increase of binding to a maximum of 82.3 ng/mg. The maximum binding achieved with apoE-1^{146} was 12.3 ng/mg, 52.0 ng/mg with apoE-$1^{127,158}$, and 19.7 ng/mg with apoE-2^{158} (**Fig. 6**). Thus, the functionally defective forms of apoE had clearly reduced capability to mediate β-VLDL binding to FH-fibroblasts. The functionally dominant mutation apoE-1^{146} showed the lowest activity among the apoE isoproteins and mutations tested.

To further investigate the interaction of apoE with polyanionic binding sites, heparin binding was used as a model for negatively charged cell surface glycoproteins. Decreased ability of apoE-1^{146} (0.25 ng apoE bound/ µg heparin) apoE-2 (0.72 ng/µg), and apoE-$1^{127,158}$ (0.47 ng/µg) to bind to heparin-Sepharose could be demonstrated, when compared to apoE-3 (1.24 ng/µg). **Table 1** summarizes the cell binding and heparin binding experiments. Defining activity of apoE-3 as 100%, the mediation of β-VLDL binding to cells by apoE-1^{146} was reduced to 18.2%, apoE-$1^{127,158}$ to 49.2%, apoE-2^{158} to 24.2%, whereas apoE-3/4 had 114% of activity. The heparin binding of apoE-1^{146} was reduced to 20.6%, apoE-$1^{127,158}$ to 37.9.2%, and apoE-2^{158} to 58.2% of apoE-3. The results of the heparin binding studies parallel the cell binding studies. In both cases the defect of apoE-1^{146} results in the most pronounced defect in the in vitro experiments.

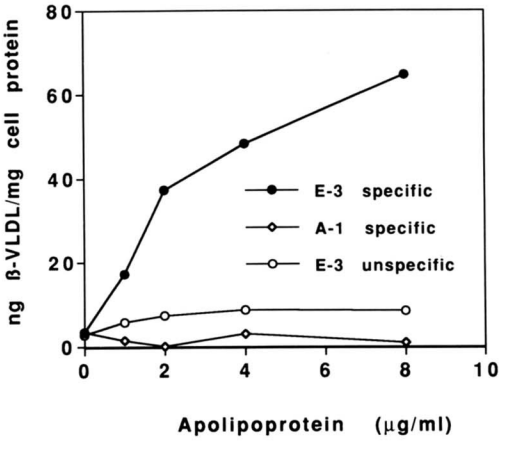

Fig. 5. Effect of apoE-3 and apoA-I on binding of β-VLDL to LDL receptor-deficient FH-fibroblasts. FH-fibroblasts (1×10^6) were incubated with ^{125}I-labeled β-VLDL (1 µg/ml) in the absence or presence of increasing amounts of apoE-3 for 1 h at 4°C. Addition of apoE-3 resulted in an increase of specific binding from 3.4 ng/mg cell protein to 64.6 ng/mg (●). The addition of apoA-I had no effect on specific binding of β-VLDL (○). Values are expressed in ng/mg cell protein and represent the mean of duplicate determinations (SD < 15%).

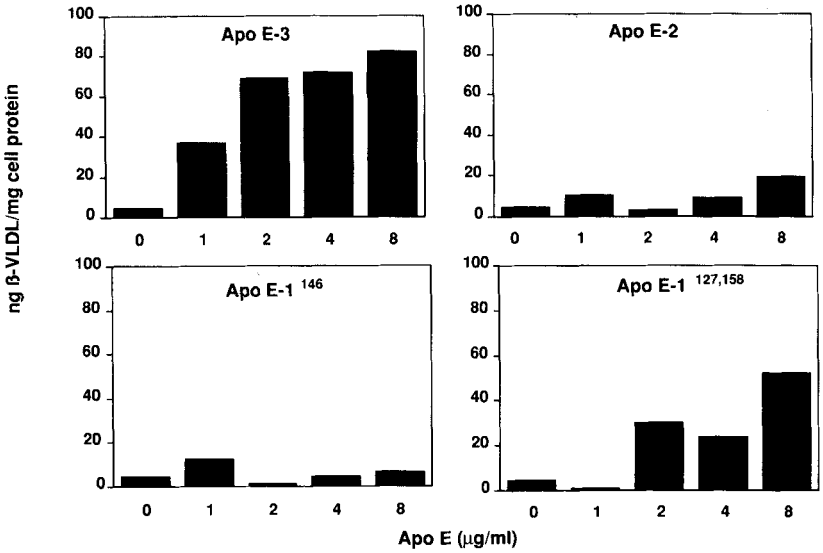

Fig. 6. Binding of β-VLDL to FH-fibroblasts: effect of apoE-1(Lys^{146}→Glu), apoE-1(Gly^{127}→Asp, Arg^{158}→Cys), apoE-2 and apoE-3. LDL receptor-deficient fibroblast cells (1 × 10^6) were incubated with ^{125}I-labeled β-VLDL (1 µg/ml) in the absence or presence of increasing amounts of the respective apoE isoform for 1 h at 4°C. Addition of apoE-3 resulted in a 13.6-fold increase of specific binding of β-VLDL in this experiment, whereas all functionally defective isoforms or mutations showed reduced capability to mediate β-VLDL binding (apoE-2: 3.2-fold increase, apoE-1^{146}: 2.1-fold increase, apoE-1^{127,158}: 8.6-fold increase). Nonspecific binding was subtracted after suppression of total binding with a 50-fold excess unlabeled β-VLDL. Bound ligand is expressed in ng/mg cell protein. Values represent the mean of duplicate determinations (SD < 15%).

DISCUSSION

ApoE is a ligand for both the LDL receptor and LRP, which has been proposed as potential chylomicron remnant receptor. LRP binds apoE among multiple other ligands (for a review, see ref. 39). The in vivo significance of the apoE-LRP interaction in humans has not yet been demonstrated; however, results in mice suggest a physiological role of LRP in remnant catabolism (7).

The aim of this study was to compare the binding of the common human apoE isoforms and two rare apoE mutations, which are associated with type III hyperlipoproteinemia, to LRP.

As a heterologous model β-VLDL was used, because it is a well-accepted model for remnant lipoproteins and is available in reproducible quality and quantity. β-VLDL is delivered to the cell by an LRP-mediated process (5), sensitive to inhibition with an anti-LRP antibody (40). In our experiments the effect of apoE on β-VLDL binding was investigated either by direct addition of apoE to the binding assay or by reassociation of apoE with β-VLDL followed by removal of free apoE. Direct addition of apoE to the assay medium increased β-VLDL binding to cells more efficiently than the β-VLDL/apoE particles. This may be explained by several reasons. Higher amounts of apoE available for binding could be achieved with direct addition, as there was no loss of apoE during the reisolation. In addition, dissociation of apoE and β-VLDL will take place during the binding assay. This equilibrium will be influenced by free apoE in the medium. Third, free apoE will bind to binding sites on the cells (i.e., proteoglycans) and could facilitate binding of β-VLDL to the cell surface. The accumulation of apoE on the cell surface has been demonstrated in the space of Disse in the liver and may, in fact, mediate the sequestration of lipoproteins (41).

Direct binding of apoE to its receptors was studied by crosslinking experiments with the chemical crosslinker EDC-NHS. In these experiments all apoE isoforms were able to bind to LRP. This is in accordance with earlier studies, where apoE-2 could be linked to LRP (4). We

TABLE 1. Binding of β-VLDL to FH-fibroblasts and heparin: effect of apoE mutations

ApoE Isoprotein	Phenotype	n[a]	β-VLDL Binding[b]	Heparin Binding[c]
			% of apoE-3	
E-3	normal	6	100 ± 30	100
E-3/4	normal	2	114 ± 12	n.d.
E-2(^{158}Arg→Cys)	variable type III	3	24 ± 6	58.2
E-1(^{158}Arg→Cys^{127}Gly→Asp)	variable type III	3	49 ± 28	37.9
E-1(^{146}Lys→Glu)	dominant type III	3	18 ± 1	20.6

[a] Number of experiments.
[b] The effect of apoE isoforms on β-VLDL binding was determined by addition of apoE (8 µg/ml) to ^{125}I-labeled β-VLDL (1 µg/ml) on FH-fibroblasts. The activity of apoE-3 to mediate β-VLDL binding was defined as 100%. Values are the mean of n experiments ± standard deviation (SD).
[c] Heparin binding was measured by heparin-Sepharose precipitation of 0.5 µg ^{125}I-labeled apoE. The binding of apoE-3 (1.24 ng/µg heparin) was defined as 100%. The SD did not exceed 15%; n.d., not determined.

also analyzed a functionally dominant mutation (apoE-1^{146}). Both apoE-1^{146} as well as the functionally recessive mutation of apoE-2^{158} are principally able to bind to LRP and thus an absolute defect in LRP binding as discriminating factor between dominant and recessive type III hyperlipoproteinemia seems unlikely. Given the complexity of the crosslinking procedure, we could only evaluate these experiments on a qualitative level. The amount of binding to LRP was measured by in vitro cell binding assays.

Addition of normal apoE-3 resulted in a 14-fold increase of β-VLDL binding to Hep3B and in an 11-fold increase to LDL receptor-deficient FH-fibroblasts. This effect was dependent on the apoE dose and highly specific, as it could be suppressed to more than 80% by excess β-VLDL. Addition of apoA-I did not have any effect on β-VLDL binding in both cell lines, and thus nonspecific protein/protein interactions, resulting in enhanced β-VLDL binding to cell surfaces, could be excluded. We further conclude that apoA-I does not enhance binding of remnant particles to LRP. In addition, ^{125}I-labeled apoA-I did not bind to LRP using chemical crosslinking (data not shown).

The ability to mediate β-VLDL binding was reduced for all apoE isoforms and mutations, which are clinically associated with type III hyperlipoproteinemia. Whereas the reduced activity of apoE-2^{158} (36% of apoE-3) on Hep3B cells may in part be explained by the presence of LDL receptors, this is excluded in the experiments using FH-fibroblasts. A reduction in mediating β-VLDL binding could be observed for apoE-2^{158} on the FH-fibroblasts. These results support and extend the data of Kowal et al. (6), who describe reduced binding of apoE-2 to LRP (ca. 40% of apoE-3) as measured by ligand blots and stimulation of cholesteryl reesterification, but not direct cell binding studies.

ApoE-1^{127,158} was found to be more efficient in mediating β-VLDL binding (49% of apoE-3) when compared to apoE-2^{158} (24% of apoE-3). This is surprising, because clinically the Gly^{127}→Asp exchange does not seem to have any relevance in addition to the Arg^{158}→Cys exchange (25). One might speculate that the Gly^{127}→>Asp exchange counteracts the conformational changes caused by the Arg^{158}→Cys exchange in the apoE molecule. However, the fact that the LDL receptor binding of both apoE-1^{127,158} and apoE-2^{158} was similarly reduced compared to apoE-3 (42) argues against a major conformational normalization induced by the Gly^{127}→Asp mutation, as this should also result in an improvement of LDL receptor binding (43).

The dominant mutation of apoE-1^{146} was found to have only 18% of the activity of apoE3, which was the lowest among the apoE isoproteins tested. The substitution of a lysine by glutamic acid at amino acid position 146 results in the substitution of a positive charge by a negative charge in the center of the LDL receptor binding domain of apoE (44). Three-dimensional analysis of the LDL receptor binding domain of apoE showed that the 146 residue is available for intermolecular interactions (44) and could be directly involved in ligand-receptor interactions. Considering the structural similarity of the ligand binding repeats in LRP and the LDL receptor, it is conceivable that the Lys^{146}→Glu mutation results in a reduction of binding to the clusters of negative charges in the LRP in analogy to the LDL receptor. These data suggest that this region also plays a central role in binding of apoE to LRP.

The difference in binding to FH-fibroblasts of both apoE-1^{146} and apoE-2 was not as pronounced, as expected for the dominant clinical manifestation of type III hyperlipoproteinemia. It would have been fascinating to correlate absent binding to LDL receptor-deficient fibroblasts with dominant remnant accumulation. Though apoE-1^{146} was found to have the lowest activity in mediating β-VLDL binding, analysis of other functionally dominant mutations should be very interesting and necessary to answer the question as to whether clinically dominant disease is correlated with reduced binding to FH-fibroblasts.

We have previously described reduced binding of apoE-1^{146} to heparin, which we used as a model for an interaction with cell surface proteoglycans (24). Though the physiological relevance of the apoE-heparin interaction in vivo remains to be established, there is evidence for a receptor-like function of cell surface glycosaminoglycans for both apoE-(45) and apoB-(46) containing lipoproteins, as well as lipoprotein lipase (LPL) (47) in vitro. Similar to apoE, LPL mediates binding of remnant particles to LRP (48), and proteoglycans appear to play an important role in this interaction (47). The general importance of proteoglycan binding is supported by reduced heparin binding of another apoE mutant (ApoE-2 Arg^{142}→Cys) described by Horie and coworkers (24). In addition, Ji et al. (49) recently showed the importance of heparan sulfate for the binding and uptake of apoE-enriched lipoproteins in cultured cells in vitro.

In the present study, reduced affinity of apoE isoforms and mutants to heparin parallels reduced ability to mediate β-VLDL binding to cells. From these data an important contribution of proteoglycans to the binding of β-VLDL seems to be indicated. Though the crosslinking experiments unequivocally show binding of all apoE isoforms to LRP, it is conceivable that this interaction requires initial immobilization of the apoE-containing particles by cell surface proteoglycans. This would not be a unique situation for lipoproteins and has been described for growth factors by several groups (50–52).

In summary, we have analyzed the interaction of the dominant mutation apoE-1^{146}, the recessive isoform apoE-2^{158}, and the variant apoE-1^{127,158}, as well as normal apoE-3 and apoE-3/4 with LDL receptor-deficient

fibroblasts and Hep3B cells. Crosslinking studies showed that all apoE proteins studied so far were able to bind to LRP. Quantitation of binding to FH-fibroblasts and to heparin showed reduced binding of all isoforms or mutations, which are clinically associated with type III hyperlipoproteinemia.

These findings underline the importance of the apoE-proteoglycan interaction. Proteoglycans seem to be responsible for the immobilization of triglyceride-rich remnant particles on cell surfaces prior to internalization. Defective proteoglycan binding thus could result in accumulation of remnant particles as observed in type III hyperlipoproteinemia. With the data presented we support the current hypothesis that proteoglycans facilitate receptor-mediated particle uptake. It remains to be clarified whether the internalization process can also be independently mediated by proteoglycans alone.

Note added in proof: During the preparation of this manuscript, Ji and coworkers presented data that are in agreement with our results by showing a correlation between reduced heparan sulfate proteoglycan binding of apolipoprotein variants and expression of type III hyperlipoproteinemia (Ji, Z. S., et al. 1994. *J. Biol. Chem.* **269:** 13421-13428).

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