Two apolipoprotein E5 variants illustrate the importance of the position of additional positive charge on receptor-binding activity

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Abstract Apolipoprotein (apo) E polymorphism has a significant effect on plasma cholesterol and low density lipoprotein cholesterol concentrations. The association of two apoE5 isoforms with elevated plasma low density lipoprotein cholesterol levels in two unrelated subjects led us to investigate the primary structures and receptor-binding properties of their apoE. Cysteamine modification and isoelectric focusing demonstrated that the apoE5 isoform from subject 1 did not contain cysteine but that the apoE5 isoform from subject 2 contained one residue of cysteine. The structural mutation in the apoE5 isoform of subject 1 was determined by peptide sequencing. Like apoE4, this variant had arginine at position 112 but differed from apoE4 by the substitution of arginine for proline at position 84. When purified and subjected to a competitive binding assay, this apoE5(84 Pro→Arg, 112 Cys→Arg) variant had the same receptor-binding activity as normal apoE3. Because subject 2 was of Japanese descent and her apoE5 contained one cysteine residue, we suspected that it would contain the lysine-for-glutamic acid mutation at position 3 that has been described previously in Japanese subjects. This was confirmed by directly sequencing the first 10 amino acid residues of her apoE. When subjected to the competitive binding assay, the total apoE from subject 2, which consisted of approximately equal amounts of normal apoE3 and apoE5(3 Glu→Lys), had a binding activity of 188%, confirming the previously reported enhanced binding of this variant. These results demonstrate that the enhancement of receptor-binding activity of more basic isoforms of apoE depends on the position at which additional positively charged amino acids are incorporated.—**Wardell, M. R., S. C. Rall, Jr., E. J. Schaefer, J. P. Kane, and K. H. Weisgraber.** Two apolipoprotein E5 variants illustrate the importance of the position of additional positive charge on receptor-binding activity. *J. Lipid Res.* 1991. **32:** 521–528.

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Through its ability to bind to cell surface receptors and mediate the uptake of lipoprotein particles, apolipoprotein (apo) E is central to the metabolism of plasma cholesterol and triglyceride (1, 2). Three common isoforms of apoE, designated apoE2, apoE3, and apoE4, occur (3), but they do not all have the same receptor-binding activities (4). Whereas the parent form, apoE3, and apoE4 bind with equal affinity to the low density lipoprotein (LDL) receptor, apoE2 possesses only ~1% of this normal receptor-binding activity (4). Apolipoprotein E4 and apoE2 differ from the parent apoE3 isoprotein only by single amino acid substitutions at positions 112 and 158 (5, 6). Whereas apoE3 has cysteine at position 112 (the only cysteine in apoE3) and arginine at position 158, apoE4 has arginine at both sites (and completely lacks cysteine) and apoE2 has cysteine at both sites (5, 6). Thus, it is the cysteine-for-arginine substitution at position 158 in apoE2 that is responsible for the dramatically decreased receptor-binding activity of this variant (4), and in subjects homozygous for apoE2, this leads to dysbetalipoproteinemia, or the accumulation of remnant lipoproteins in the plasma (7, 8). Most of these subjects have plasma and LDL cholesterol levels that are slightly lower than normal (9, 10), but they are at a markedly increased risk of developing type III hyperlipoproteinemia, which is characterized by severely elevated cholesterol and triglyceride levels (11).

Although apoE4 has normal receptor-binding activity (4), it is nevertheless associated with elevated plasma and LDL cholesterol levels (10, 12). The mechanism responsible for this is unknown; however, relative to apoE3, apoE4

Abbreviations: apo, apolipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein; PVDF, polyvinylidene difluoride; SDS, sodium dodecyl sulfate; DMPC, dimyristoylphosphatidylcholine.

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does preferentially distribute to triglyceride-rich lipoproteins [chylomicrons and very low density lipoproteins (VLDL)] (13, 14), and the positively charged arginine at position 112 has been implicated in this process (15). The relative enrichment of apoE on the chylomicron and VLDL particles is thought to lead to their accelerated uptake and the consequent down-regulation of LDL receptor expression. This, in turn, would account for the increased levels of LDL (the major cholesterol-carrying lipoprotein) in the plasma of apoE4 subjects. Through these effects of apoE2 and apoE4, the apoE gene accounts for a significant fraction of the variation in plasma cholesterol levels in the population. It has been estimated that 60% of the variation in plasma cholesterol levels is genetically determined and that apoE polymorphism accounts for approximately 14% of the genetic variation (10).

In addition to the three common apoE isoforms, a number of rare apoE mutants have been described (for review, see ref. 2) and in one family apoE deficiency has been described (16). Because many of these natural mutants were associated with lipoprotein abnormalities and had defective receptor-binding activities, they helped define a region of the molecule in the vicinity of residues 130–160, rich in basic amino acids, that is very important for receptor binding. Prediction of the secondary structure of this region indicates that residues 131–150 form an α-helix, with many of the basic residues occupying one face of the helix (6). Natural as well as bacterially produced mutants in which subsitutions for these basic residues occur resulted in reduced receptor-binding activity; it is thought that these basic residues are responsible for forming ionic interactions with negatively charged residues in the ligand-binding domain of the LDL receptor (2, 17).

Whereas the removal of positive charge from this region of apoE results in a decrease of binding activity, a bacterially produced variant that had an additional positively charged residue introduced into the basic α-helix (arginine for serine at position 139) resulted in enhanced binding activity (18). A natural variant with enhanced receptor-binding activity, apoE5(3 Glu→Lys), has also recently been reported (19), and this also involved the addition of positive charge to the molecule (20). However, the mechanism by which the addition of positive charge near the amino terminus enhances receptor binding is unknown. In this report we describe the molecular abnormalities in two apoE5 variants that are associated with elevated LDL cholesterol levels. One of these contains the same mutation previously reported (20), apoE5(3 Glu→Lys), and we confirmed the enhanced receptor-binding activity of this variant. The second is a novel apoE5 variant with normal receptor-binding activity. The differential effects of these mutations support the hypothesis that the position of additional positively charged amino acids is critical to the alteration of receptor-binding activity.

METHODS

Subject description

Subject 1 was a 54-year-old woman of European descent with the apoE5/3 phenotype whose plasma and lipoprotein lipid levels have been described previously (21). She had both elevated plasma total cholesterol (266 mg/dl) and LDL cholesterol (177 mg/dl) levels. Subject 1 had no claudication, no chest pain upon exertion, and no evidence of either cardiovascular or cerebrovascular disease. Her mother, age 75 years, also has the apoE5/3 phenotype and elevated plasma total cholesterol (308 mg/dl) and LDL cholesterol (208 mg/dl) levels and also has no evidence of angina, coronary heart disease, claudication, or stroke. There were no secondary causes of elevated LDL, such as thyroid disease, liver disease, kidney disease, or diabetes mellitus present in either subject 1 or her mother, and the elevated lipid levels in both subjects were reduced significantly with a combination of cholesterol-lowering diet and lovastatin treatment.

Subject 2 was a 64-year-old woman of Japanese descent who also has the apoE5/3 phenotype. She had familial hypercholesterolemia with tendon xanthomas and LDL cholesterol levels consistently above 250 mg/dl. She also had very severe triple-vessel disease as determined by angiography.

Isolation and characterization of apolipoprotein E

Lipoproteins of $d < 1.006$ g/ml, $d < 1.02$ g/ml, and $d < 1.21$ g/ml were prepared from the subjects' plasma using EDTA as anticoagulant. Total apoE was isolated as described previously (4, 22). Apolipoprotein E isoforms were isolated in two ways. Preparative Immobiline isoelectric focusing was performed on 5% polyacrylamide slab gels containing 6 M urea and having a pH gradient of 5.5–6.5 (23). Thiopropyl chromatography was used to separate apoE isoforms containing cysteine from those that did not and was performed as described (24). Analytical isoelectric focusing was performed on 5% polyacrylamide gels containing 8 M urea and 2% Ampholine, pH 4–6 (Pharmacia LKB Biotechnology Inc.), according to the method of Pagnan et al. (25) with the modification previously described (26). The apoE on the $d < 1.02$ g/ml lipoproteins was modified with cysteamine as described (4).

Structural analysis

Purified apoE isoforms were digested with L-(1-tosylamido-2-phenyl) ethyl chloromethyl ketone trypsin

(Cooper Biomedical, Freehold, NJ), and the tryptic peptides were separated by two-dimensional paper mapping as described (27); 0.5 mg of digest was applied to each map. Tyrosine-containing peptides were located using a specific staining procedure (28). Amino acid analyses were performed using two systems. Phenylthiocarbamyl amino acid analyses (29) were performed on all peptides from the two-dimensional paper maps using a Waters Nova-Pak C_{18} stainless steel column (15 cm × 3.9 mm, Waters Chromatography Division, Millipore Corp., Milford, MA) maintained at 34°C on a Beckman model 334 High Performance Liquid Chromatography system (Beckman Instruments, Fullerton, CA). For the intact protein, amino acid analyses were performed on a Beckman 121MB analyzer as described previously for apoE (5). Cysteine was determined as cysteic acid according to the method of Moore (30).

Automated peptide sequencing was performed on an Applied Biosystems 477A pulsed-liquid sequencer (Applied Biosystems, Foster City, CA) equipped with an online 120A analyzer. Tryptic peptides that had been located on the paper maps with fluorescamine (Aldrich Chemical Co., Milwaukee, WI) were eluted with acetic acid–ethanol–water 1:1:8 (v/v/v) and sequenced. Because limited quantities of apoE were available from subject 2, the amino-terminal sequence of her total apoE was determined directly on an Immobilon polyvinylidene difluoride (PVDF) membrane (Millipore Corp., Bedford, MA) for 10 cycles. For this procedure, a volume of d<1.006 g/ml lipoproteins that contained 150 μg of total protein was delipidated once with chloroform–methanol 2:1 (v/v). The apolipoprotein mixture was then dissolved in 3% sodium dodecyl sulfate (SDS), 10% glycerol, 2% β-mercaptoethanol, 62.5 mM Tris-HCl, pH 6.8, and separated by SDS-polyacrylamide gel electrophoresis on a 10–20% gradient slab gel using the buffer system of Laemmli (31) but substituting 2-amino-2-methyl-1,3-propanediol for Tris. Immediately after electrophoresis, the proteins were transferred onto the PVDF membrane using a semi-dry electrophoretic transfer unit (Gelman Sciences) for 1 h at 350 mA. The proteins were located with Ponceau S, and the pieces of membrane containing apoE were placed in the reaction cartridge of the sequencer for analysis.

Receptor binding assays were carried out using apoE–dimyristoylphosphatidylcholine (DMPC) complexes as competitors for ^{125}I-labeled LDL binding to cultured human fibroblasts (32).

RESULTS

Electrophoretic analysis of apolipoprotein E

Isoelectric focusing analysis of the apolipoproteins in the d<1.006 g/ml lipoprotein fraction from fasting plasma demonstrated that both subjects 1 and 2 had the apoE5/3 phenotype (**Fig. 1**, lanes 1 and 5). However, after cysteamine treatment the apoE5 isoform of subject 1 did not shift, indicating that it did not contain cysteine (lane 2), whereas the apoE5 isoform of subject 2 shifted one charge unit to the E6 position (lane 6), indicating that it contained one cysteine residue. As expected, the apoE3 isoform in both subjects also moved one charge unit more cathodally, to the E4 position (lanes 2 and 6).

Structural analysis

Because the apoE5 isoform in subject 2 contained one residue of cysteine and because she was of Japanese descent, we suspected that this apoE variant might be identical to the apoE5 variant found in the Japanese population, i.e., apoE5(3 Glu→Lys) (20). To test this, we performed direct protein sequencing of total apoE from subject 2 that had been transferred onto a PVDF membrane from an SDS-polyacrylamide gel of the d<1.006 g/ml lipoproteins (fasting VLDL) from this subject. The sequencing results for 10 cycles (**Table 1**) show that both glutamic acid and lysine were detected at the third cycle. The glutamic acid is from the normal apoE3 isoform in this subject, whereas the lysine is from the variant apoE5 isoform, where it arises as a result of a mutation of 3 Glu→Lys, the same substitution previously described (20). Based on a repetitive yield of 85% (calculated from the yields of the valines at cycles 2 and 6 and the glutamic acid residues at cycles 7 and 9; this value for repetitive yield is typical when sequencing samples from PVDF membranes), the yields of the amino acids determined at cycle 3 indicate that of the total apoE in the VLDL of sub-

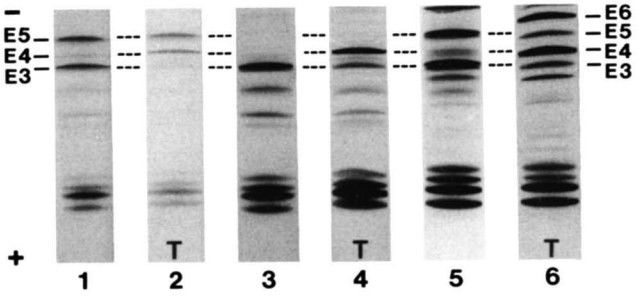

Fig. 1. Analytical isoelectric focusing of the very low density lipoproteins from subjects 1 and 2. Lane 1, 150 μg of untreated d<1.02 g/ml lipoproteins from subject 1; lane 2, 150 μg of cysteamine-treated (T) d<1.02 g/ml lipoproteins from subject 1; lane 3, 150 μg of untreated d<1.02 g/ml lipoproteins from a subject homozygous for normal apoE3; lane 4, 150 μg of cysteamine-treated (T) d<1.02 g/ml lipoproteins from a subject homozygous for normal apoE3; lane 5, 150 μg of untreated d<1.006 g/ml lipoproteins from subject 2; lane 6,150 μg of cysteamine-treated (T) d<1.006 g/ml lipoproteins from subject 2. The cathode (−) is at the top, and the anode (+) is at the bottom. The focusing positions of apoE3, apoE4, apoE5, and apoE6 are indicated. The minor bands migrating in the E4 position in lanes 1 and 5 are the monosialylated apoE5 variants from subjects 1 and 2, respectively.

TABLE 1. Amino-terminal sequence analysis of total apolipoprotein E from subject 2, demonstrating the substitution at position 3

Cycle	Amino Acid Determined	Concentration
		pmol
1	Lys	8.5
2	Val	22.9
3	{ Glu	12.2
	{ Lys	2.4
4	Gln	26.1
5	Ala	29.2
6	Val	14.5
7	Glu	17.6
8	Thr	19.8
9	Glu	17.0
10	Pro	10.3

ject 2, ~60% is the apoE3 isoform and ~40% is the variant apoE5 isoform.

The apoE5 isoprotein in subject 1 was shown not to contain cysteine by isoelectric focusing and therefore represents a new apoE variant. We purified this isoform by preparative Immobiline focusing in a pH gradient of 5.5–6.5. The isoelectric point of the apoE5 isoform from this subject was determined to be ~6.2, and the purity of the isoform was assessed by isoelectric focusing (**Fig. 2**, lane 3). The apoE5 isoform was also purified from the total apoE of subject 1 by thiopropyl chromatography. By this method, the apoE3 isoform is retained by the column through interaction of the cysteine sulfhydryl with the functional thiol groups of the column resin, while the apoE5 isoform, which does not contain cysteine, does not bind and flows directly through the column.

The results of amino acid analysis of both apoE isoforms from subject 1 are given in **Table 2**. The results for the apoE5 isoform are the mean of five determinations of this isoform prepared either by Immobiline focusing or thiopropyl chromatography. The results for the apoE3 isoform are the mean of nine determinations of this isoform prepared either from subject 1 by both methods or from a subject homozygous for apoE3. Three significant differences between the apoE3 and apoE5 isoforms were indicated by the amino acid analyses. First, whereas apoE3 had one cysteine (as indicated by the isoelectric focusing results), it was confirmed that the apoE5 isoform did not contain cysteine. Second, the apoE5 isoform had an additional 1.6 residues of arginine compared with apoE3. The third difference observed was that the apoE5 isoform had one less proline residue than apoE3. These results were consistent with the apoE5 isoform of subject 1 possessing arginine rather than cysteine at residue 112 and additionally having an arginine-for-proline substitution somewhere else in the molecule.

To determine the latter substitution site in this apoE5 isoform, the apoE5 was digested with trypsin and subjected to two-dimensional paper mapping. There were four new peptides present on the apoE5 tryptic peptide map (**Fig. 3**) as compared with the tryptic map of normal apoE3. Three of these new peptides had a net negative charge, and one had a net positive charge. One of the new acidic peptides and the new basic peptide, labeled T13* and T13**, respectively (Fig. 3), arose as a result of an arginine-for-cysteine substitution at position 112, yielding a new tryptic cleavage site in apoE5. The presence of the other two acidic peptides (labeled T8/9* and T9** in Fig. 3) also arose as a result of a new tryptic cleavage site. Peptide T8/9* stained positively for tyrosine, and its amino acid composition is given in **Table 3**. The sequence of this new peptide was Ala-Tyr-Lys-Ser-Glu-Leu-Glu-Glu-Gln-Leu-Thr-Arg. Inspection of this sequence indicated that it represented amino acid residues 73–84 of normal apoE, but with arginine substituted for the normally occurring proline-84. The other acidic peptide (labeled T9** in Fig. 3) had the composition given in Table 3 and the sequence Val-Ala-Glu-Glu-Thr-Arg, which represents amino acid residues 85–90 of normal apoE. Therefore, peptides T8/9* and T9** result from the substitution of arginine for proline at residue 84, introducing a new tryptic cleavage site in the apoE5 variant. This also means that, although this apoE5 variant has arginine at position 112, making it "E4-like," it focuses in the E5 position because of the presence of the additional positively charged arginine residue at position 84.

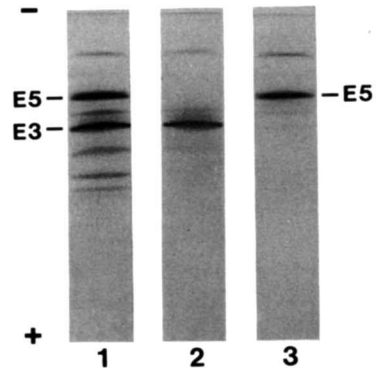

Fig. 2. Analytical isoelectric focusing of purified apoE3 and apoE5 isoforms from subject 1. Lane 1, 10 μg of the total apoE from subject 1; lane 2, 5 μg of the apoE3 isoform from subject 1; lane 3, 5 μg of the apoE5 isoform from subject 1. The purified apoE3 and apoE5 isoforms in lanes 2 and 3 had been isolated by preparative Immobiline focusing and were subsequently used for structural determinations. The cathode (–) is at the top, and the anode (+) is at the bottom. The focusing positions of apoE3 and apoE5 are indicated.

TABLE 2. The amino acid compositions of the purified apolipoprotein E3 and apolipoprotein E5 isoforms from subject 1

Amino Acid	E3 (n = 9)	E5 (n = 5)	Δ[a] E5–E3
Lys	12.4 (12)[b]	12.7	+0.3
His	2.1 (2)	2.3	+0.2
Arg	32.2 (34)	33.8	+1.6*
Cys	N.D. (1)	0.1	−0.9*
Asx	13.2 (12)	13.1	−0.1
Thr	10.7 (11)	10.5	−0.2
Ser	12.6 (14)	12.8	+0.2
Glx	71.3 (71)	71.6	+0.3
Pro	8.6 (8)	7.7	−0.9*
Gly	17.9 (17)	17.4	−0.5
Ala	34.9 (35)	35.3	+0.4
Val	22.3 (22)	22.3	0
Met	5.8 (7)	5.8	0
Ile	2.3 (2)	2.3	0
Leu	37.6 (37)	37.3	−0.3
Tyr	3.7 (4)	3.6	−0.1
Phe	3.1 (3)	3.3	+0.2
Trp	N.D. (7)	N.D.	
Total residues	299		

N.D., not determined.

[a]The values determined for amino acids in the apoE3 isoform were subtracted from the values determined for amino acids in the apoE5 isoform. These results suggested that the apoE5 isoform had gained two arginines at the expense of one cysteine and one proline relative to apoE3 (indicated by asterisks).

[b]The numbers in parentheses were determined by sequencing (6).

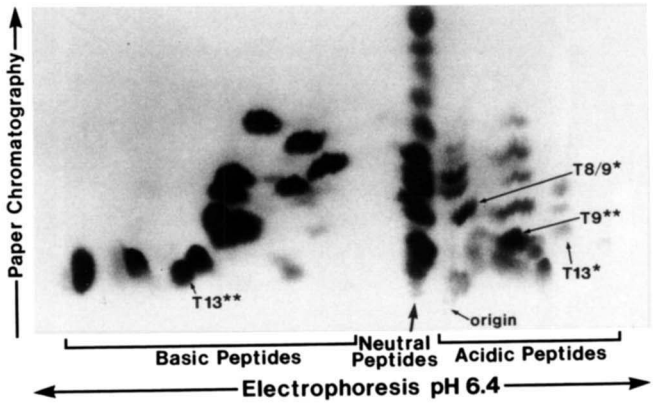

Fig. 3. Fluorescamine-stained two-dimensional tryptic peptide map of the purified apoE5 isoform from subject 1. The tryptic digest (0.5 mg) was applied at the origin and electrophoresed at 3 kV for 55 min in a pH 6.4 pyridine–acetate buffer (pyridine–acetic acid–water 100:4:900, v/v/v) in the first dimension and chromatographed in the ascending direction in the second dimension for 20 h in the upper phase of pyridine–isoamyl alcohol–water 6:6:7 (v/v/v) (27). Basic, neutral, and acidic peptides are indicated. The peptides are designated as in Rall et al. (6) and Wardell et al. (37). The new peptides in this apoE5 isoform are labeled with asterisks and result from the introduction of two new tryptic cleavage sites in this variant.

Receptor-binding activities of the apolipoprotein E5 variants from subjects 1 and 2

Receptor binding assays were performed on the isolated apoE5 isoform from subject 1 that had either been purified from the total apoE fraction by preparative Immobiline focusing (only the nonglycosylated apoE5) or by thiopropyl chromatography (nonglycosylated plus glycosylated apoE5). In total, four binding experiments indicated that the receptor-binding activity of this variant, apoE5(84 Pro→Arg, 112 Cys→Arg), was normal. A concentration of 30.0 ± 7 ng of apoE5(84 Pro→Arg, 112 Cys→Arg)/ml was required to prevent 50% of the ^{125}I-labeled LDL from binding to the LDL receptors of cultured human fibroblasts, which was not significantly different from 35.9 ± 8 ng/ml for normal apoE3 (n = 8) (unpaired *t*-test). Data from a representative binding experiment are plotted in **Fig. 4**. There were no differences in the binding activities of the apoE5 preparations that had been isolated by the two different procedures described above, indicating that glycosylation did not affect receptor-binding activity.

Because limited amounts of plasma were available from subject 2, we were able to obtain only a very small amount of her apoE. Therefore, receptor binding assays were performed on the total mixture of the two components, normal apoE3 and apoE5(3 Glu→Lys), isolated from her d<1.21 g/ml lipoproteins and present in approximately equal amounts. In three separate binding assays the apoE3/5-DMPC complexes were consistently more active in binding to the LDL receptors on cultured human fibroblasts when compared with normal apoE3. A concentration of 19.1 ± 8 ng of apoE3/5(3 Glu→Lys)/ml was able to compete for 50% of the binding of ^{125}I-labeled LDL to the LDL receptor, which was significantly different ($P < 0.02$) from a concentration of 35.9 ± 8 ng/ml

TABLE 3. Amino acid compositions of selected tryptic peptides of apolipoprotein E from subject 1

Amino Acid	T13*[a]	T13**	T8/9*	T9**
Asx	1.9 (2)[b]			
Glx	1.1 (1)		4.3 (4)	2.2 (2)
Ser	0.1	0.1	0.9 (1)	
Gly	1.2 (1)	0.8 (1)	0.4	
His				
Arg	0.9 (1)	1.1 (1)	1.0 (1)	0.8 (1)
Thr			0.6 (1)	0.7 (1)
Ala	1.0 (1)	0.2	1.1 (1)	1.1 (1)
Pro				
Tyr			0.7 (1)	
Val	1.0 (1)		0.4	1.0 (1)
Met	0.9 (1)			
Ile				
Leu	0.9 (1)	0.2	2.2 (2)	
Phe				
Lys			0.9 (1)	
Total residues	9	2	12	6

[a]The peptides are designated as in Rall et al. (6) and Wardell et al. (27).

[b]The numbers in parentheses were determined by sequencing.

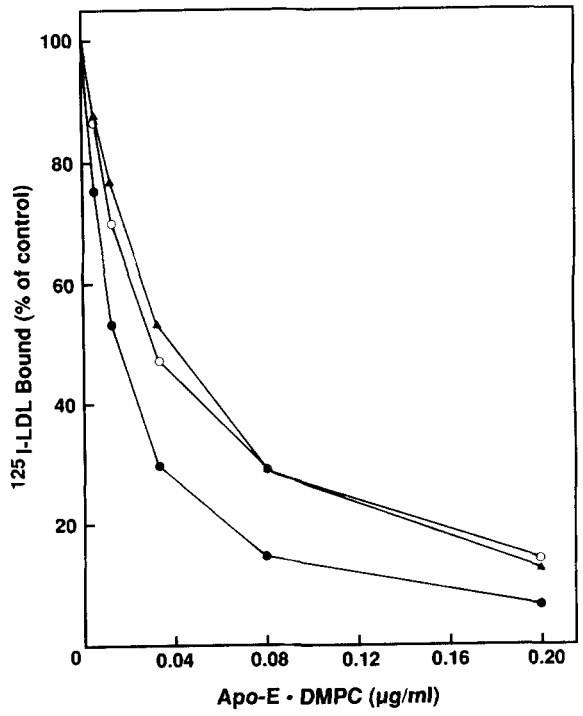

Fig. 4. Receptor-binding activities of the purified apoE5 isoform from subject 1 and the total apoE from subject 2. Various concentrations of apoE-DMPC complexes were mixed with 2 μg of ^{125}I-labeled LDL and incubated with cultured human fibroblasts on 35-mm culture dishes for 4 h at 4°C. (○) Normal apoE3; (▲) apoE5(84 Pro→Arg, 112 Cys→Arg) purified from subject 1; (●) total apoE isolated from subject 2, who is heterozygous for normal apoE3 and apoE5(3 Glu→Lys).

for normal apoE3 (n = 8) (unpaired *t*-test). A representative binding assay for this variant is given in Fig. 4. Based on these data, the total apoE from subject 2, who possessed the apoE5(3 Glu→Lys) isoform, displayed 188% of normal binding. Previously, Dong, Yamamura, and Yamamoto (19) reported that the purified apoE5(3 Glu→Lys) isoform was two times more active in binding to LDL receptors than normal apoE3.

DISCUSSION

The primary structures of two different apoE5 variants have been determined. The first, apoE5(84 Pro→Arg, 112 Cys→Arg), was present in a 54-year-old woman of European descent (subject 1) with elevated LDL cholesterol levels who was part of the Framingham Offspring Study. Despite her elevated LDL cholesterol levels, subject 1 was totally asymptomatic, with no evidence of either cardiovascular or cerebrovascular disease. This was also true for her mother, who also had the apoE5/3 phenotype and elevated LDL cholesterol. Furthermore, there is no clear history of premature heart disease in this family. Because the apoE5 variant in subject 1 had arginine at position 112, it is likely that apoE5(84 Pro→Arg, 112 Cys→Arg) arose as a result of a point mutation, CCG to CGG, in the ε4 allele. The second variant, apoE5(3 Glu→Lys), was present in a 64-year-old woman of Japanese descent (subject 2) who had familial hypercholesterolemia and atherosclerotic vascular disease. This variant has previously been reported to result from a single base substitution, GAG to AAG, in the ε3 allele (20), has only been observed in subjects of Japanese origin, and appears to be commonly associated with mild types of hyperlipidemia in which LDL levels are elevated (19, 33). The E5 isoelectric focusing position of both of these variants is explained by the above results; in apoE5(84 Pro→Arg, 112 Cys→Arg) by the presence of two additional positively charged arginine residues compared with apoE3, and in apoE5(3 Glu→Lys) by a double charge mutation. Because we performed only partial amino acid sequencing, it is possible that other mutations involving noncharged, frequently occurring amino acids might also exist, although this seems unlikely.

The apoE5 variant from subject 1, apoE5(84 Pro→Arg, 112 Cys→Arg), was shown to possess normal receptor-binding activity, which means that the binding activity is not altered by the addition of positive charge at either position 112 [as is already known for apoE4 (4)] or position 84, or at both. It would appear that the substitution of arginine for proline at position 84 is functionally silent. In contrast, as previously reported by Dong et al. (19) and confirmed in this study, the substitution of a positively charged lysine for glutamic acid at position 3 results in an apoE isoform with enhanced receptor-binding activity. The previous investigators reported that the purified apoE5(3 Glu→Lys) isoform was two times more active in binding to LDL receptors than normal apoE3 (19). They speculated that this may lead to an increased delivery of apoE-containing lipoproteins to the liver and a consequent down-regulation of LDL receptors, accounting for the elevated LDL cholesterol. Our binding studies determined that an approximately equal mixture of apoE5(3 Glu→Lys) and normal apoE3 possessed 188% of normal binding activity. The apparent difference in the binding activity obtained by Dong et al. (19) using the purified apoE5(3 Glu→Lys) isoform and that reported here using the total apoE from subject 2 is probably due to differences in experimental procedures.

Because the more basic forms of apoE have been associated with increased plasma and LDL cholesterol levels, it has been suggested that the total positive net charge on apoE variants plays an important role in cholesterol metabolism and the development of atherosclerosis (34, 35). Tajima et al. (34) also speculated that the substitution of two lysine residues for two glutamic acid residues in apoE7-Suita(244 Glu→Lys, 245 Glu→Lys), the most basic apoE isoform reported to date, may introduce another LDL receptor-binding domain

into the molecule, and they suggested that hyperlipoproteinemia associated with more basic apoE isoforms may result from altered receptor-binding activities. However, our results support the hypothesis that it is not increased net positive charge per se that results in enhanced receptor-binding activity (and therefore elevated cholesterol levels), but rather the mutation of specific residues at critical positions in the apoE molecule.

The enhanced receptor-binding activity of apoE5(3 Glu→Lys) is rather difficult to interpret. This mutation occurs in a region of the molecule that has not been shown to be functionally important. However, it is possible that the substitution of lysine for glutamic acid at position 3 in apoE influences receptor binding indirectly, as has been shown for arginine-158 (36) and the 7-amino acid insertion in apoE3-Leiden (37). It is noteworthy that the amino terminus of apoE is highly charged and certainly has the potential to interact with other charged residues in the molecule. It is presumably the change in the charge of the amino terminus in apoE5(3 Glu→Lys) that leads to the enhancement of receptor binding in this variant, but the mechanism is as yet unclear. In this respect, it will be interesting to learn whether another recently reported apoE variant that also has a mutation that changes the net charge of the amino terminus by +2, apoE5(13 Glu→Lys) (38), also has enhanced receptor-binding activity. Whatever the mechanism involved in apoE5(3 Glu→Lys), these results support the hypothesis that enhancement of receptor-binding activity occurs only when positive charge is added at critical positions in the structure of apoE.

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